contamination of food, and that antibodies should be useful in preventing the disorder. What was not known until Dr. Tzipori was able to conduct studies in gnobiotic pigs, was that it is the Shiga-like toxin II that is the principle cause of HUS, whereas organisms that express only Shiga-like toxin I, or even to some extent, Shiga-like toxin I and II, are more likely to cause disease that does not have such devastating sequela. As shown in the excerpt entitled "Disease Overview" from Mr. Hefferman's power point presentation, most cases of *E. coli* result in bloody diarrhea one to eight days following exposure. This technology is aimed at preventing those cases where HUS develops, with multi-system morbidity, including death (3-5%), chronic renal failure, neurological complications, and other systemic complications (50%).

Unlike the prior art which used *in vitro* studies in cell culture to test toxin neutralization with antibodies, Dr. Tzipori determined that it was possible to infect pigs, who have an intestinal system much like humans. Mice cannot be infected, so one can only look at neutralization of toxin. He then compared the symptoms of animals infected with different strains of the *E. coli*, to see what toxin(s) caused what symptoms. This allowed one to determine what toxin(s) to target, and how much antibody would be required. It also allowed one to determine that it was possible to administer antibody after infection, since this is the case one is most likely to have in real life. Since this application was originally filed, a number of groups have now demonstrated that it is the Shiga-like toxin II that is the major cause of HUS in humans. See, for example, the enclosed representative articles: Boerlin, et al., "Associations between Virulence Factors of Shiga Toxin-Producing *Escherichia coli* and Disease in Humans" J. Clin. Microbiol. 37(3), 497-503 (1999); Bielaszewka, et al. "Isolation and characterization of sorbitol-

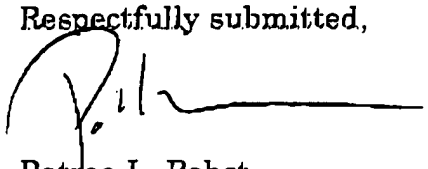
Association with Clinical Symptoms" J. Infectious Dis. 185:74-84 (2002). When strains produce only Shiga-like toxin I, there is no HUS. See, for example, in addition to the above articles, Hashimoto, et al. "Epidemic of grastrointestinal tract infection including hemorrhagic colitis attributable to Shiga toxin 1-producing *Escherichia coli* O118:H2 at a junior high school in Japan Pediatrics 103(1):e2 (1999).

There is currently no approved product for treating these patients. Therefore, the claimed composition is critically important for patients, most of whom are small children, who develop this life threatening disease.

The claims have been amended to limit the method of use to a composition consisting of antibodies which neutralize shiga-like toxin II in an amount effective to treat or prevent HUS. Claims to the method of use of a combination of antibodies to both shiga-like toxin II and I will be pursued in another another. A preliminary amendment has been filed in the related case, U.S.S.N. 10/041,958, with the composition claims from this application, canceled solely to facilitate prosecution by narrowing issues.

Allowance of claims 1, 4-6, and 27-32, is earnestly solicited.

Respectfully submitted,



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COLLEGIUM Pharmaceutical

Disease Overview

Shiga Toxin E.Coli Exposure

Incidence: 1-2.6/10,000 (100,000 U.S)

Bloody Diarrhea 1-8 days from exposure

Recovery

Hemolytic Uremic Syndrome (HUS)

Incidence: 5-10%
Median Age: 5 y/o
(5,000-10,000 U.S)

Multi-System morbidity Occurs w/in 1 week of diarrhea

Death
Incidence: 3-5%
(150-500 U.S)

Chronic Renal Failure
Neurological complications
Other System complications
Incidence: 50%
(2,500-5,000 U.S.)

Recovery- Long term Complications ?

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Associations between Virulence Factors of Shiga Toxin-Producing *Escherichia coli* and Disease in Humans

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Associations between known or putative virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans were investigated. Univariate analysis and multivariate logistic regression analysis of a set of 237 isolates from 118 serotypes showed significant associations between the presence of genes for intimin (*eae*) and Shiga toxin 2 (stx_2) and isolates from serotypes reported in humans. Similar associations were found with isolates from serotypes reported in hemorrhagic colitis and hemolytic-uremic syndrome. The enterohemorrhagic *E. coli* (EHEC) hemolysin gene was significantly associated with isolates from serotypes found in severe diseases in univariate analysis but not in multivariate logistic regression models. A strong association between the intimin and EHEC-hemolysin genes may explain the lack of statistical significance of EHEC hemolysin in these multivariate models, but a true lack of biological significance of the hemolysin in humans or in disease cannot be excluded. This result warrants further investigations of this topic. Multivariate analysis revealed an interaction between the *eae* and stx_2 genes, thus supporting the hypothesis of the synergism between the adhesin intimin and Shiga toxin 2. A strong statistical association was observed between the stx_2 gene and severity of disease for a set of 112 human isolates from eight major serotypes. A comparison of 77 isolates of bovine origin and 91 human isolates belonging to six major serotypes showed significant associations of the genes for Shiga toxin 1 and EspP protease with bovine isolates and an increased adherence on HEp-2 cell cultures for human isolates, particularly from diarrheic patients and healthy persons.

Shiga toxin-producing *Escherichia coli* strains (STEC) were first implicated in disease in the early 1980s by their association with hemolytic-uremic syndrome (HUS) and hemorrhagic colitis (HC) (16, 27). STEC have subsequently been associated with uncomplicated diarrhea (23) and have been isolated from stools of healthy individuals. STEC are now considered a major cause of disease in developed countries (10, 17). HC usually begins with abdominal cramps and diarrhea, followed by bloody diarrhea. HUS patients present with acute renal failure, thrombocytopenia, and microangiopathic hemolytic anemia, often following a prodromal diarrhea. HC and HUS are severe diseases which frequently require hospitalization, and HUS may be fatal in up to 5% of cases. STEC infections are mainly food borne, and bovine feces are the main source of food contamination by this organism (10). A large variety of STEC serotypes have been implicated in human disease, but some STEC serotypes found in cattle or in food have never or only very rarely been associated with severe human disease. These apparent differences in STEC serotype frequencies may, in part, be due to methodological issues, but differences in the ability of STEC strains to cause disease are also likely contributors.

Based on in vitro and animal model studies, several virulence factors have been described in STEC, the major one being Shiga toxins (11). Two main categories of Shiga toxins have been distinguished. *E. coli* Shiga toxin 1 (Stx1) is almost identical to the Shiga toxin of *Shigella dysenteriae* in amino acid sequence and cannot be distinguished from it serologically, whereas Shiga toxin 2 (Stx2) is less related to the Shiga toxin of *Shigella* and is not neutralized by antibodies to either Stx1 or Shiga toxin from *S. dysenteriae* (21, 35). As is the case with enteropathogenic *E. coli*, some STEC strains can tightly attach to epithelial cells of the intestine through an adhesin called intimin. Such strains induce in the underlying cells profound structural modifications called attaching and effacing lesions. The genes related to these lesions, including the *eae* (for *E. coli* attaching and effacing) gene, which encodes intimin, are clustered in a pathogenicity island named the locus for enterocyte effacement (LEE [19]). Recently, Schmidt and collaborators reported the genetic analysis of a new plasmid-encoded hemolysin of STEC called enterohemorrhagic *E. coli* hemolysin (EHEC hemolysin; *ehxA* gene), which seemed to be associated with severe clinical disease in humans (31, 32). A protease (EspP), encoded by the same plasmid as EHEC hemolysin, has also recently been described in some STEC serotypes and has been suggested as an additional virulence factor of STEC (5). There is actually no experimental proof for the role of EHEC hemolysin and EspP in the virulence of STEC. They are therefore only putative virulence factors, but for the sake of simplicity, they will be included with the other virulence factors for the remainder of the discussion.

Previous studies have shown a large diversity in the distribution of virulence factors among STEC strains (1, 3, 15, 41). Associations have been suggested between the presence of some of these factors in STEC and their virulence (24, 29, 30, 32). However, these studies were often relatively small scale or examined the distribution of each virulence factor separately, without accounting for possible associations between virulence factors and without considering the rest of the genome of the bacterial pathogen. In the present study, the distribution of virulence factors in an international collection of STEC iso-

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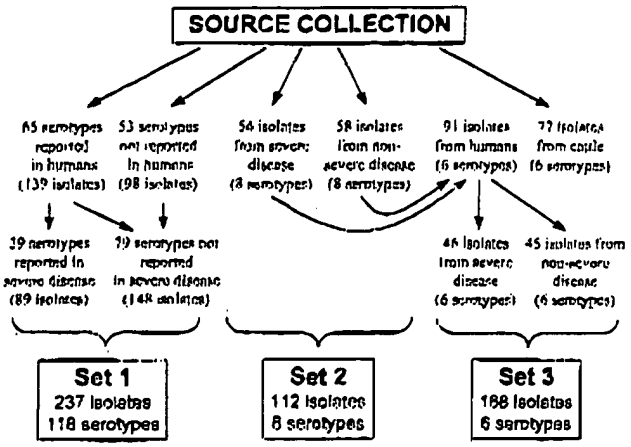

FIG. 1. Graphical representation of sampling strategy used to obtain sets 1 to 3 of STEC isolates.

lates representing a broad spectrum of serotypes from various sources was determined and analyzed by methods which account for these possible influences. The first aim of the study was to determine associations between virulence factors and STEC disease in humans, based on classification of STEC isolates by serotypes reported or not reported in the literature to have been isolated from humans. Multivariate analysis was used to control for the confounding effects of other virulence factors and of the genomic background of the isolates by using serotype as a proxy. The second aim was to examine the diversity of virulence factors in serotypes most frequently associated with disease and to detect associations between any of these factors and the severity of disease in the actual patients from whom the isolates were recovered. The last aim of this study was to compare bovine and human STEC populations of the major serotypes involved in human disease to test whether human STEC from these serotypes that are most commonly isolated from patients with disease form a different population than the bovine STEC population of the same serotypes.

MATERIALS AND METHODS

STEC isolates. Three different sets of STEC isolates were used for the present study (Fig. 1). The first set comprises 237 STEC isolates of 118 serotypes originating from humans (n = 60), animals (n = 159), and food (n = 18) that were selected from a larger collection of STEC isolates deposited at the Health of Animals Laboratory, Health Canada, Guelph, Ontario, Canada. Stratified random sampling (strata are equivalent to serotypes) was used for the selection in order to represent all the serotypes available in the collection. The number of isolates per serotype was limited to a maximum of three, and for those serotypes with less than three isolates in the collection, all were used. The source collection is the fruit of a long-term effort to collect representative STEC isolates from human and nonhuman sources of diverse geographic origin. Based on an extensive review of the literature, the isolates were classified into four categories (Fig. 1). The first category within set 1 (listed below) comprised 139 isolates belonging to 65 serotypes previously reported in humans (O1:H20, O2:H5, O2:H6, O2:H27, O2:H29, O5:H−, (nonmotile isolates) O6:H−, O7:H4, O8:H14, O15:H27, O15:H−, O22:H8, O22:H16, O26:H11, O26:H−, O38:H21, O45:H2, O48:H21, O55:H7, O55:H9, O75:H1, O76:H19, O80:H−, O82:H8, O84:H2, O89:H−, O91:H10, O91:H14, O91:H21, O91:H−, O98:H−, O103:H2, O111:H8, O111:H−, O112:H2, O113:H4, O113:H7, O113:H21, O114:H4, O115:H18, O117:H4, O118:H12, O118:H16, O118:H30, O119:H6, O119:H−, O121:H19, O126:H8, O126:H21, O128:H2, O128:H−, O132:H−, O145:H−, O146:H8, O146:H21, O153:H25, O157:H7, O157:H−, O163:H19, O163:H−, O165:H25, O165:H−, O171:H2, O172:H−, and OX3:H21). The second category comprised 98 isolates of 53 serotypes not previously reported in humans (O2:H39, O2:H−, O5:H11, O6:H10, O6:H34, O8:H8, O8:H9, O8:H16, O8:H19, O8:H35, O15:H7, O22:H2, O39:H49, O40:H8, O43:H2, O46:H38, O46:H−, O49:H−, O69:H11, O76:H25, O77:H39, O84:H−, O85:H−, O88:H25, O91:H7, O98:H25, O110:H8, O111:H11, O113:H−, O115:H8, O116:H21, O118:H−, O119:H5, O119:H25, O121:H7, O126:H27, O128:H35, O130:H38, O132:H18, O136:H12, O136:H16, O136:H−, O139:H19, O142:H38, O145:H8, O153:H21, O153:H31, O156:H7, O156:H8, O156:H25, O156:H−, O163:H2, and O168:H8). The third category is a subset of the first one and comprised 89 isolates belonging to 39 serotypes clearly identified in the literature as associated with HUS and HC (underlined in the first list above). The fourth category of isolates within set 1 corresponds to the remaining 148 isolates of 79 serotypes not previously reported in severe human disease. No significant difference between categories in terms of mean number of isolates per serotype was detected by a z test. The overall mean number of isolates per serotype was 2.008. This supports our attempt to control for serotype confounding in set 1 at the sampling level.

The second set (Fig. 1 and Table 1) comprises 112 epidemiologically unrelated isolates of human origin belonging to eight serotypes often associated with human disease. These isolates represent all the human isolates of these serotypes from the STEC collections deposited at the Health of Animals Laboratory, Health Canada, Guelph, for which suitable clinical information was available. They originate from Belgium (n = 53), Germany (n = 17), Switzerland (n = 16), the United States (n = 14), Canada (n = 6), Australia (n = 3), and Denmark (n = 3). Based on clinical information available from the donors (Table 1), these isolates were further classified into two categories. The first category (nonsevere disease) comprises isolates from healthy persons and from patients with uncomplicated nonbloody diarrhea. The second category (severe disease) comprises isolates from patients with bloody diarrhea or from patients with clinical signs of HUS.

The third set of isolates (Fig. 1 and Table 1) comprises all the human isolates from six of the eight serotypes of set 2 and identical numbers of randomly chosen isolates of bovine origin of the same serotypes. For serotypes for which there were fewer isolates of bovine origin than of human origin, all the bovine isolates were used.

Detection of stx_1, stx_2, *eae*, *ehxA*, and *espP*. All the isolates were examined for the presence of the stx_1 and stx_2 genes by PCR under the conditions described by Pollard and collaborators (25) except for three isolates for which the Congenic primers and conditions were used (26). The STEC strains EC9100/94 (serotype O46:H38; bovine origin) and 4304 (serotype O157:H7; human origin) served as positive controls, and the enteropathogenic *E. coli* strain 2348/69 served as a negative control for this test. The presence of *eae* was detected by PCR under the conditions described by Sandhu and coworkers (30) and was confirmed by dot blot hybridization when necessary. Strains 4304 and JM109 (43) served as positive and negative controls, respectively. For the dot blot hybridization, the probe consisted of the digoxigenin-labeled PCR product of strain 4304 produced with the DIG DNA labeling and detection kit (Boehringer, Mannheim, Germany). Cell lysates were obtained by resuspending the cells of a 500-μl overnight culture in Luria-Bertani broth in 100 μl of 0.4 M NaOH and heating it for 30 min at 80°C. One microliter of cell lysate was blotted on a Hybond membrane (Amersham Life Science, Little Chalfont, England) and bound by UV cross-linking.

TABLE 1. Numbers of STEC isolates of human and bovine origin in strain sets 2 and 3 classified by serotype

Isolate source[a]	No. of isolates								
	O26:H11	O26:H−	O103:H2	O111:H8	O111:H−	O145:H−	O157:H7	O157:H−	Total
NS	5	0	1	0	0	1	0	3	10
D	5	7	9	1	10	1	12	3	48
BD	1	2	5	1	1	1	13	0	24
HUS	4	2	1	0	4	3	12	4	30
Total human	15[b]	11	16[b]	2[b]	15[b]	6[b]	37[b]	10	112
Bovine	8[b]	0	16[b]	2[b]	13[b]	6[b]	32[b]	0	77

[a] NS, STEC isolates from humans with no symptoms; D, isolates from humans with uncomplicated diarrhea; BD, isolates from humans with bloody diarrhea; HUS, isolates from humans with hemolytic-uremic syndrome. All of the human isolates were used for set 2.

[b] Isolate used for set 3.

TABLE 2. Overall distribution of *ehxA*, *espP*, *eae*, stx_1, and stx_2 in STEC isolates from serotypes which are reportedly not found in humans, from serotypes found in humans, and from serotypes clearly associated with severe disease in humans (set 1)

Gene	Distribution[a] in serotypes:			
	Total	Not from humans	From humans	From severe disease
ehxA	146 (61.6)	59 (60.2)	87 (62.6; 0.7100)	64 (71.9; 0.0114)
espP	148 (62.4)	60 (61.2)	88 (63.3; 0.7441)	55 (61.8; 0.8728)
eae	77 (32.5)	23 (23.5)	54 (38.8; 0.0128)	43 (48.3; 0.0001)
stx_1	148 (62.4)	72 (73.5)	76 (54.7; 0.0033)	46 (51.9; 0.0358)
stx_2	134 (56.5)	42 (42.9)	92 (66.2; 0.0004)	63 (70.8; 0.0006)
No. of isolates	237	98	139	89
No. of serotypes	118	53	65	39

[a] Of the total 237 isolates of set 1, 159 were of animal origin, 18 were of food origin, and 60 were of human origin. The numbers in the columns represent numbers of isolates. The first number in parentheses indicates the percentage of the total category positive for the respective characteristic. The second number in parentheses for the fourth column indicates the *P* value for chi-square tests comparing isolates from serotypes found in humans with those not found in humans. The second number in parentheses in the last column indicates the *P* value for chi-square tests comparing isolates from serotypes associated with severe disease to all the others.

Hybridization was done following standard protocols (28) with stringent washing at 65°C in 0.2× SSC (20× SSC is 3 M NaCl plus 0.3 M sodium citrate, pH 7.0). Probe that remained bound to homologous sequences was detected with the DIG DNA labeling and detection kit following the supplier's instructions. The presence of *ehxA* was detected by PCR following the method of Sandhu and collaborators (29). Strains 4304 and 2348/69 served as positive and negative controls, respectively. Expression of the hemolytic phenotype was detected by incubating isolates overnight on washed sheep erythrocyte plates (2). When PCR and phenotype results were contradictory, dot blot hybridization of plasmid DNA was used to confirm the results with a digoxigenin-labeled probe made of the *ehxA* PCR product of strain 4304 as described by Beutin and collaborators (4). Plasmid preparations were made by the alkaline lysis method (28) with one phenol-chloroform extraction, and 1 μl of each preparation was blotted onto Hybond membrane and bound by UV cross-linking. Hybridization and detection were done under the same conditions as for *eae*. For detection of *espP*, the same dot blot plasmid hybridization method was used as for *ehxA*. The digoxigenin-labeled probe consisted of a PCR product from strain 4304 covering the whole *espP* coding sequence. Strains 4304 and 2348/69 served as positive and negative controls, respectively. In case of questionable results, the detection was repeated by using Southern blotting (28) after running 15 μl of plasmid preparation in a 0.8% agarose gel. Hybridizations after Southern blotting were done as described above for dot blots. The presence of the genes was used as a proxy for the proteins they encode.

Adherence of STEC on HEp-2 cell cultures. HEp-2 cell adherence assays were performed according to standard protocols (7, 20). Briefly, 5×10^4 HEp-2 cells were incubated overnight in 400 μl of Eagle's minimum essential medium (EMEM) with antibiotic and 10% fetal calf serum (FCS) in each well of an eight-well permanox culture slide (Nalge Nunc International Naperville, Ill.) at 37°C in a 5% CO_2 atmosphere. The cells were washed three times with phosphate-buffered saline (PBS; pH 7.2) before use. The STEC strains to be tested were cultivated under aerobic conditions overnight at 37°C in Luria-Bertani broth and subcultured in EMEM with 10% FCS and 1 mM $CaCl_2$ at 37°C overnight in a 5% CO_2 atmosphere. Approximately 4×10^6 bacteria were inoculated in 300 μl of EMEM with 10% FCS and 1% D-mannose in each well of the culture slides and incubated for 3 h at 37°C in a 5% CO_2 atmosphere. The wells were washed three times with PBS, and the cells were incubated for 3 more hours under the same conditions. The slides were then washed three times with PBS, and the cells were stained with the Diff-quick staining kit (Dade Diagnostics Inc, Aguada, Puerto Rico) following the instructions of the manufacturer. A total of 200 cells were examined under the microscope, and the cells with more than 10 adherent bacteria per cell were counted. Strain 6-264 (O157:H7) was used as a positive control for each batch of tests. To control for day-to-day variation, all the results were reported in proportion to the positive control. To control for within-day variations, all tests were done in duplicate, and the results are expressed as the average of the duplicates.

Statistical analysis. All analyses were performed with SAS for Windows version 6.12 (SAS Institute Inc., Cary, N.C.). For the analysis of associations between virulence factors and isolates of serotypes associated with humans or of serotypes known to be involved in severe disease (set 1), univariate analysis with chi-square tests (34) and multivariate analysis with logistic regression, including a backward-elimination procedure (threshold of 5% significance), were used (14). Associations between covariates were analyzed with McNemar's association tests (34). Reproducibility of the HEp-2 cell adherence assay was assessed by calculating an intracluster correlation coefficient (34) with a generalized linear model. To test for potential interactions between *eae* and the genes of the other factors in the logistic regression model, a manual forward procedure was used with a threshold of 5% significance (statistical interactions are present when two explanatory variables do not act independently on a response variable, thus suggesting the presence of synergism or antagonism at the biological level). The same procedures were used for the analysis of associations between virulence factors and severity of disease in isolates of human origin (set 2). However, for the latter analysis, the serotype variables were forced into the model, the level of adherence on HEp-2 was also included, and the *eae* variable was not used because all the isolates under study were positive for this characteristic. Finally, the same approach was used for the comparison of virulence factors and adherence level in STEC isolates of six major STEC serotypes of human origin versus those of bovine origin and of isolates from severe or less severe human disease versus those of bovine origin (set 3).

RESULTS

Homogeneity of virulence factors within serotypes (set 1). All the isolates within a serotype were identical in terms of presence or absence of the *eae* gene for the 65 serotypes with more than one isolate in set 1. Within these 65 serotypes, the *ehxA* and *espP* genes were consistently present or absent in 54 and 52 serotypes, respectively. The variability in terms of Shiga toxin was slightly higher, with 43 and 41 serotypes with homogeneous patterns for stx_1 and stx_2, respectively. A strong association was present between *eae* and *ehxA* in the McNemar test ($P < 0.0001$; odds ratio [OR] = 9.3).

Associations between virulence factors and isolates of serotypes reported in humans (set 1). The distribution of the genes for the virulence factors under study in the different categories of set 1 is presented in Table 2. The results of the chi-square tests for the comparison of isolates from serotypes found in humans and those from serotypes not found in humans are reported in the fourth column of Table 2. When modeling the associations among the five virulence factors encoded by *ehxA*, *espP*, *eae*, stx_1, and stx_2 and presence in humans with a logistic regression model, only *eae* and stx_2 appeared as significant variables. This was the case in both a full logistic model comprising all the virulence factors as independent variables and in a reduced model resulting from the backward-elimination procedure. The only significant interaction between intimin and the other virulence factors of STEC at the 5% level was between Eae and Stx2. The coefficients, corresponding ORs, and *P* values for the two models with and without interaction are reported in Table 3 and 4.

Associations between virulence factors and isolates of serotypes reported in severe human disease (set 1). The proce-

TABLE 3. Coefficients, *P* values, and ORs for the logistic regression models of the association between virulence factors and STEC isolates from serotypes reported in humans[a]

Gene	Model 1			Model 2		
	β	*P*	OR	β	*P*	OR
ehxA	--	--	--	--	--	--
espP	--	--	--	--	--	--
eae	1.08	0.0008	2.93	0.55	0.1719	1.74
stx_1	--			--	--	--
stx_2	1.23	0.0001	3.41	0.86	0.0114	2.36
*eae**stx_2	--	--	--	1.72	0.0473	NA

[a] The first model was obtained by using a backward-elimination procedure with a threshold of 5% significance. The second model was derived from the first one by using a forward procedure with a threshold of 5% significance to detect significant two-way interaction terms. β, coefficient; *eae**stx_2, interaction between *eae* and stx_2; --, variables not significant at the 5% level and not included in the final models; NA, not applicable.

TABLE 4. Detailed ORs for the logistic regression model of the association between virulence factors and STEC isolates from serotypes reported in humans, including the *eae***stx*$_2$ interaction (set 1)[a]

Comparison	OR
eae positive *stx*$_2$ negative vs *eae* negative *stx*$_2$ negative	1.74
eae negative *stx*$_2$ positive vs *eae* negative *stx*$_2$ negative	2.36
eae positive *stx*$_2$ positive vs *eae* positive *stx*$_2$ negative	13.20
eae positive *stx*$_2$ positive vs *eae* negative *stx*$_2$ positive	9.68
eae positive *stx*$_2$ positive vs *eae* negative *stx*$_2$ negative	22.87

[a] *eae* positive, isolate carrying the *eae* gene; *eae* negative, isolate lacking the *eae* gene; *stx*$_2$ positive, isolate carrying the *stx*$_2$ gene; *stx*$_2$ negative, isolate lacking the *stx*$_2$ gene. These results indicate, for instance, that an *eae*-positive and *stx*$_2$-positive STEC isolate is 13.2 times more likely to be from a serotype previously reported in humans than an *eae*-positive but *stx*$_2$-negative isolate.

dures described above were also used to compare isolates from serotypes found in severe human disease with those from other serotypes. The results of the chi-square tests for this comparison are reported in the last column of Table 2. The logistic regression analysis of these data resulted in a model similar to the previous one, with only *eae* (coefficient = 1.67; P value = 0.0001; OR = 5.33) and *stx*$_2$ (coefficient = 1.58; P value = 0.0001; OR = 4.86) significantly associated with isolates from serotypes found in severe disease. However, there was no evidence to suggest an interaction between intimin and the toxins.

Reproducibility of the HEp-2 cell adherence assay. Based on repeated trials of the HEp-2 cell adherence assays with a set of 15 isolates representing a broad range of adherence levels (0 to 1.4 in proportion to the positive control), an intracluster correlation coefficient of 0.89 was obtained. This result shows that 89% of the variability observed in the HEp-2 cell adherence assays is due to the strains and that only 11% of the variability is due to experimental error.

Associations between virulence factors of human STEC isolates from eight major serotypes and disease severity (set 2). The distribution of the genes for the virulence factors under study in the different categories of isolates from set 2 and the corresponding P values for the chi-square tests are presented in Table 5. Among the variables tested (serotype, *ehxA*, *espP*, *stx*$_1$, and *stx*$_2$, and level of adherence on HEp-2 cell cultures), *stx*$_1$ was associated with uncomplicated diarrhea and healthy individuals in the univariate analysis (Table 5) and *stx*$_2$ was significantly associated with severe disease in both the univariate analysis and the multivariate logistic regression models (coefficient = 1.60; P = 0.0038; OR = 4.95). There was no evidence ($P > 0.05$) to suggest an interaction between the level of adherence on cell cultures and the toxins.

Comparison of distribution of virulence factors between isolates of human and bovine origin among six common STEC serotypes (set 3). The distribution of the genes for the virulence factors under study and the level of adherence on HEp-2 cell cultures are presented in Table 6. Univariate analysis with chi-square tests suggests a clear association between *stx*$_1$ and bovine isolates compared to that with human isolates in general (P value < 0.008). This crude analysis also suggests the same association when comparing isolates of bovine origin with those from humans with severe disease. In addition, STEC isolates from patients with uncomplicated diarrhea and healthy individuals seem to adhere significantly better to HEp-2 cells than do isolates from cattle. Logistic regression confirms the association between *stx*$_1$ and bovine isolates, when compared to human isolates in general, to isolates from patients with severe STEC-associated disease, or to isolates from patients with uncomplicated diarrhea and healthy individuals (Table 7). Logistic regression analysis shows similar associations for *espP* and a significantly higher level of adherence on HEp-2 cells for STEC from humans in general, and particularly for STEC from patients with uncomplicated diarrhea and healthy individuals when compared to bovine isolates (Table 7). Finally, the logistic regression models also suggest a lower prevalence of *stx*$_2$ among isolates from patients with simple diarrhea and healthy individuals than among those from cattle.

DISCUSSION

Among over 100 serotypes that have been recovered from humans, serotypes O157:H7 and O157:H− clearly represent the majority of isolates associated with disease. However, STEC organisms of many other serotypes have been isolated from patients with HUS and HC with variable frequencies. The differences in frequencies may be partially related to reagent availability and methodological bias in the detection of STEC (9). However, previous studies have also shown a large spectrum of variability in virulence factor makeup in STEC populations, and many researchers have attempted to correlate the presence of specific recognized or putative virulence factors with disease or severity of disease (10, 12, 17, 22, 24, 29, 30, 32, 33, 38, 39). The main conclusion of these previous investigations has been that no single factor is responsible for the virulence of STEC. In all these studies, the role of each factor has been analyzed separately, without accounting for linkages between virulence factors. This simple approach may bias estimates of the role of putative virulence factors in disease pathogenesis by not correcting for the con-

TABLE 5. Overall distribution of virulence factors in 112 human STEC isolates of serotypes O26:H11, O26:H−, O103:H2, O111:H8, O111:H−, O145:H−, O157:H7, and O157:H− isolated from individuals with severe disease or with either uncomplicated diarrhea or no symptoms (set 2)

Gene or characteristic	Distribution[a]		
	Total (n = 112)	HUS and HC (n = 54)	D and NS (n = 58)
ehxA	101 (90.2)	50 (92.6; 0.4075)	51 (87.9)
espP	86 (76.8)	42 (77.8; 0.8104)	44 (75.9)
eae	112 (100.0)	54 (100.0; 1.000)	58 (100.0)
stx$_1$	75 (67.0)	29 (53.7; 0.0040)	46 (79.3)
stx$_2$	60 (53.6)	39 (72.2; 0.0001)	21 (36.2)
HEp-2[b]	1.144 (SD = 0.810)	1.056 (SD = 0.696; 0.2662)	1.227 (SD = 0.901)

[a] BD, bloody diarrhea; D, uncomplicated diarrhea; NS, no symptoms. The first number in parentheses indicates the percentage of the total category positive for the characteristic. The second number in parentheses for the third column indicates the P value for a chi-square test comparing isolates from severely diseased patients with those from patients with uncomplicated diarrhea and healthy individuals.

[b] HEp-2, level of adherence on HEp-2 cell cultures measured in proportion to a positive control.

TABLE 6. Overall distribution of virulence factors in 168 human and bovine STEC isolates of serotypes O26:H11, O103:H2, O111:H8, O111:H−, O145:H−, and O157:H7 (set 3)

Gene or characteristic	Distribution[a]				
	Overall (n = 168)	Bovine (n = 77)	Human (n = 91)	HUS + BD (n = 46)	D + NS (n = 45)
ehxA	158 (94.0)	73 (94.8)	85 (93.4; 0.7026)	45 (97.8; 0.4117)	40 (88.9; 0.2277)
espP	140 (83.3)	69 (89.6)	71 (78.0; 0.0446)	37 (80.4; 0.0224)	34 (75.6; 0.0389)
eae	168 (100.0)	77 (100.0)	91 (100.0; 1.0000)	46 (100; 1.0000)	45 (100; 1.0000)
stx_1	129 (76.8)	67 (87.0)	62 (68.0; 0.0039)	26 (56.5; 0.0001)	36 (80.0; 0.3027)
stx_2	83 (49.4)	37 (48.1)	46 (50.5; 0.7470)	33 (71.7; 0.0103)	13 (28.9; 0.0378)
HEp-2[b]	0.991 (SD = 0.741)	0.847 (SD = 0.557)	1.112 (SD = 0.852; 0.0203)	1.004 (SD = 0.682; 0.1667)	1.223 (SD = 0.992; 0.0083)

[a] The first number in parentheses indicates the percentage of the total category positive for the respective characteristic. The second number in parentheses for the last three columns indicates the P value for the comparisons with the isolates of bovine origin in chi-square tests.
[b] HEp-2, level of adherence on HEp-2 cell cultures measured in proportion to a positive control.

founding effect of other virulence factors and by neglecting joint effects as observed in the case of synergistic mechanisms.

The *eae* gene and the entire LEE can be spread horizontally in STEC populations. However, this event seems to be rare, and the presence of the LEE is strongly associated with particular STEC lineages (4). The *ehxA* and the *espP* genes are carried on the same plasmid (5) and are therefore physically linked. Recent work in our laboratory suggests some associations between the LEE, the EHEC hemolysin plasmid, and the hemolysin itself (4). Previous studies have shown a certain degree of homogeneity for the presence of virulence factors within STEC serotypes (12, 29, 30), and the results of the present study confirm this observation. This is also true, although at a slightly lower level, for the phage-encoded (35) Shiga toxin genes. Analysis of *E. coli* populations by use of multilocus enzyme electrophoresis (6) has shown that serotype is a good marker for evolutionary lineages and is therefore also likely to be a good marker for the genetic background of STEC in terms of unknown virulence factors involved in the pathogenesis of STEC-associated diseases. Altogether, these data strongly support the approach taken in the present study, in which we tried to control for the confounding effects of the above-described genetic links among virulence factors and between virulence and serotype, an approach not used in previous works.

The first part of our study examined the association between the virulence factors of STEC and isolates from serotypes found in humans or in severe disease. Data on the exact origins of STEC isolates received in microbiological laboratories and reference collections are often very sparse, in particular with regard to clinical information. To overcome this limiting factor, we chose to use for the first part of the study a classification of isolates based on serotypes and their respective associations with humans as stated in the literature. Due to a lack of exhaustive descriptions and reporting in the literature, this approach may be subject to misclassification. It is expected that if this type of misclassification occurs, it will tend to decrease the significance of potential associations. Therefore, our approach may tend to ignore some weak but otherwise significant associations.

We observed no major difference in the frequency of *ehxA* and *espP* between isolates from serotypes found in humans and those not found in humans (Table 2). However, *eae* and stx_2 were significantly more frequent in isolates from serotypes found in humans, and this association was even more significant when we compared isolates from serotypes clearly associated with severe disease to isolates from other serotypes. The reverse is true for stx_1, which seems to be found more frequently among isolates from serotypes not found in humans than among those associated with humans. A significant difference in *ehxA* frequency was observed between isolates from serotypes specifically associated with severe disease and those that are not. This is not the case for *espP*. These crude data suggest an association of *eae* and stx_2 with isolates of serotypes found in humans and possibly of *eae*, stx_2, and *ehxA* with severity of disease (Table 2). Our results are in agreement with those of previous studies showing that *ehxA* (29, 32), *eae* (24), and stx_2 (22, 33, 37) are found more frequently in STEC isolates from patients with severe disease than in other STEC populations and that stx_1 may be associated with some STEC isolates of bovine origin (18, 40). However, in our multivariate analysis, only *eae* and stx_2 were significantly associated with isolates from serotypes found in humans or with isolates from serotypes implicated in severe human disease. This suggests that most of the crude association of EHEC hemolysin with severe disease could be due to confounding effects of the major virulence factor intimin (Fig. 2A). Alternatively, collinearity between *eae* and *ehxA* in our model may obscure the true relationship between *ehxA* and human STEC isolates or disease (Fig. 2B). Thus, as has been shown by others (2, 29, 31, 32), our results confirm that EHEC hemolysin may represent an interesting virulence marker for STEC involved in severe human disease. How-

TABLE 7. Coefficients, P values, and ORs in logistic regression models describing associations between STEC virulence factors and origin of STEC isolates (human with severe disease or uncomplicated diarrhea and healthy individuals versus bovine)[a]

Gene or characteristic	Model 1			Model 2			Model 3		
	β	P	OR	β	P	OR	β	P	OR
ehxA	–	–	–	–	–	–	–	–	–
espP	−1.56	0.0094	0.21	−1.43	0.035	0.14	−1.60	0.0282	0.20
stx_1	−1.78	0.0006	0.17	−1.94	0.0006	0.24	−2.20	0.0046	0.11
stx_2	–	–	–	–	–	–	−1.88	0.0435	0.15
HEp-2	0.95	0.0047	2.59	–	–	–	1.22	0.0045	3.39

[a] Model 1, logistic regression model obtained by backward-elimination procedure and describing associations between STEC virulence factors and human isolates in comparison to bovine isolates; model 2, logistic regression model obtained by backward-elimination procedure and describing associations between virulence factors and isolates from severe disease in comparison to bovine isolates; model 3, logistic regression model obtained by backward-elimination procedure and describing the association between virulence factors and isolates from patients with uncomplicated diarrhea and healthy individuals in comparison to bovine isolates. The backward-elimination procedure had a threshold of 5% significance. HEp-2, level of adherence on HEp-2 cell cultures measured in proportion to a positive control; β, coefficient; –, variables not significant at the 5% level and not included in the model. The results indicate, for instance, that when all the other factors are kept constant, an *espP*-positive isolate is five times less likely (equivalent to 0.20 times more likely) to originate from a healthy human or a human with simple diarrhea than from cattle (model 3). Similarly, for each increase of 100% in the adherence level (in comparison to the positive control), an isolate is 2.59 times more likely to originate from humans than from cattle (model 1).

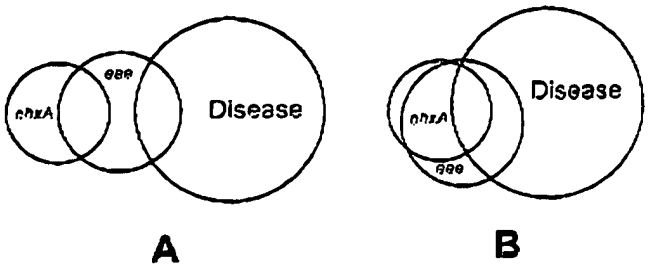

FIG. 2. Conceptual models of associations between *ehxA*, *eae*, and disease resulting in statistical association between *ehxA* and disease in univariate analysis but not in multivariate analysis (for reasons of simplicity, stx_2 was not included in these graphical representations). (A) Confounding effect of *eae*, i.e., strong association of *eae* with disease, no direct association between *ehxA* and disease, and strong association of *ehxA* with *eae*. (B) Collinearity of *eae* and *ehxA*, i.e., strong association of *eae* with disease, strong association of *ehxA* with disease, and strong association of *ehxA* with *eae*.

ever, our results also suggest that the role that EHEC hemolysin plays in the virulence of STEC may not be a major one, and therefore a reevaluation of its involvement in pathogenesis of severe disease due to STEC is warranted. Experimental work on animal and in vitro models is needed to clarify this point.

Adherence of STEC on enterocytes may be a necessary step for persistent colonization of the human intestine and for an efficient local delivery of toxins, allowing a significant absorption of Shiga toxins in or through enterocytes and more severe effects on the organism than would have occurred without adherence. It is therefore not surprising that our analysis shows an association between *eae* and isolates from serotypes found in humans and, moreover, one of our logistic regression models also suggests a possible interaction between *eae* and stx_2. It would be of interest, therefore, to confirm this finding on a larger collection of STEC with precisely defined origins.

The second part of our study concentrated on a few serotypes frequently associated with disease and evaluated the association of virulence factors within these serotypes with the severity of disease. Our results show a high prevalence of *eae* and *ehxA* in STEC isolates of these serotypes regardless of disease severity (Table 5). This confirms the observation made in the first part of the study suggesting an important role of intimin in strains from serotypes involved in disease and an association between the *eae* and *ehxA* genes in STEC populations. One observes a striking difference in prevalence of stx_1 and stx_2 between isolates from patients with severe disease and isolates from patients with simple diarrhea and healthy individuals. When we perform a logistic regression analysis (forcing the serotypes into the model), our results show a strong association between stx_2 and severe disease: an stx_2-positive isolate is approximately five times more likely to be associated with severe disease than an stx_2-negative isolate of the same serotype. In view of the results of the first part of the study, this conclusion is not surprising, and it fits with suggestions made by others using animal models (8, 36, 39) or less extensive epidemiological studies based on serogroup O157 only (22, 33, 37). No other factor reaches a significant level of association with severe disease in the logistic regression analysis. Interestingly, our full logistic regression model suggests a positive but statistically nonsignificant association between EHEC hemolysin and severity of disease (data not shown). Due to the high prevalence of EHEC hemolysin and low diversity in the population, only the analysis of a much larger number of isolates would allow us to confirm this association and to obtain a valid estimate of its coefficient. However, the results of the first part of this study suggest that this coefficient would probably be relatively low. The observed level of STEC adherence on HEp-2 cell cultures did not show any significant association with severity of disease in the univariate or the multivariate logistic regression analysis for this part of the study.

The third part of our study used STEC isolates of six major serotypes frequently involved in disease to examine if STEC isolates of these serotypes isolated from humans may form a different population than those from the bovine STEC reservoir. Trends visible in the univariate analysis (Table 6) are confirmed by multivariate analysis (Table 7) and show a significant association of stx_1 and *espP* with bovine STEC populations of these serotypes. They also show that human isolates of these *eae*-positive serotypes adhere more strongly on HEp-2 cell cultures than those from cattle. This fact is particularly marked in the case of isolates from patients with diarrhea or from healthy carriers and suggests that increased adherence on epithelial cells may play a role in the pathogenesis of STEC-associated diarrhea. Our results from cell cultures are in agreement with another report (38) suggesting that adherence may be a more important factor in STEC-associated diarrhea than Shiga toxins. However, our results should be confirmed with other cell lines more representative of polarized enterocytes (42) or in more complex systems, like the recently described adherence tests on organ cultures (13). These models may be more relevant for assessment of the adherence of STEC. They may better mimic the in vivo conditions encountered by STEC in the human bowel, thus allowing the bacteria to fully express characteristics only poorly expressed on HEp-2 cell cultures.

In conclusion, our results formally show that intimin and Stx2 are the virulence factors of STEC that are most strongly associated with disease in humans, and particularly with severe disease. These results suggest that STEC strains carrying the *eae* and stx_2 genes should be the main targets of preventive and therapeutic measures. We could not detect any significant association of the newly described EspP protease with disease in humans. In contrast with previous studies using univariate analysis, the present work using multivariate analysis did not show any significant association between EHEC hemolysin and disease. This may be due either to a true lack of biological significance of EHEC hemolysin in the pathogenesis of STEC-associated diseases or to collinearity problems in multivariate modeling. The latter point clearly needs further clarification. Our results show that distribution of virulence factors and adherence levels differ between human and animal populations of the same serotypes. Thus, our results strongly suggest that STEC isolates from humans form a different population than those found in the bovine reservoir or that they are only a subpopulation of the latter.

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Escherichia coli Harboring Shiga Toxin 2 Gene Variants: Frequency and Associati n with Clinical Symptoms

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Shiga toxin (Stx)–producing *Escherichia coli* (STEC) from patients with hemolytic-uremic syndrome (HUS), patients with diarrhea without HUS, or asymptomatic subjects were genotyped to assess associations between stx_2 variants and clinical manifestations of infection. Neither stx_{2d} nor stx_{2e} was found in 266 STEC isolates from patients with HUS. Of 262 STEC isolates from patients with diarrhea, stx_{2d} was found in 41 (15.6%; $P < .000001$), and stx_{2e} was found in 12 (4.6%; $P = .0004$). The stx_2 genotype frequency was similar among isolates from patients with HUS (3.7%) and diarrhea (5.0%). The frequencies of stx_{2c}, stx_{2d}, and stx_{2e} among 96 STEC isolates from asymptomatic subjects were comparable to those among isolates from patients with diarrhea. None of the 626 STEC isolates contained stx_{2f}. All stx_{2e}-positive or stx_2-positive STEC isolates were eae negative and originated from subjects older than those with STEC isolates with stx_1, stx_{2c}-positive STEC isolates can cause HUS, but the presence of stx_{2d} or stx_{2e} may predict a milder disease with a minimal risk of HUS.

Shiga toxin (Stx)–producing *Escherichia coli* (STEC) cause diarrhea, hemorrhagic colitis, and hemolytic-uremic syndrome (HUS) worldwide [1–5]. Stx are considered to be the cardinal virulence factors of STEC. These toxins consist of 2 major types, Stx1 and Stx2 [6]. Stx2 is closely related to a family of Stx2 variants or alleles (Stx2c [7], Stx2d [8], Stx2e [9], and Stx2f [10]). These respective Stx2 variants have 99.7%, 94.9%, 94.0%, and 63.4% nucleotide sequence identity in their A subunits and 95.2%, 86.6%, 79.0%, and 75.4% nucleotide sequence identity in their B subunits to the corresponding subunits of the Stx2-encoding gene [7–10]. Although Stx2c and Stx2d are produced by STEC strains isolated from humans [7, 8, 11–14], Stx2e typically is associated with pig edema disease [9, 15] and has been detected only rarely in STEC of human origin [16–19]. Stx2f has been identified in STEC isolated from feral pigeons [10], but, to date, only a single human isolate [20] has been shown to possess an stx_2 variant with extensive (>99%) nucleotide sequence homology to stx_{2f} [10].

Data suggest that the clinical outcome of STEC infection depends on the *stx* genotype of the infecting strain. Although the stx_1 or stx_1/stx_2 genotypes predominate in STEC isolated from patients with uncomplicated infection (i.e., those who do not develop HUS) [21, 22], stx_2 has been the most prevalent toxin genotype identified in STEC isolated from patients with HUS [21, 22]. In a study from the United States, patients infected with STEC O157 possessing stx_2 but not stx_1 were significantly more likely to develop systemic sequelae, including HUS, than were patients infected with STEC O157 harboring stx_1 alone or stx_1 and stx_2 [22]. Recently, a strong statistical association also was demonstrated between the presence of the stx_2 genotype and the severity of human disease, including the development of HUS and bloody diarrhea for STEC belonging to the major non-O157 serogroups, including O26, O103, O111, and O145 [23]. These data demonstrate that the stx_2 genotype augments the ability of STEC to cause serious human diseases [22, 23]. In contrast to STEC containing stx_2, the association of STEC that harbor *stx* variants with clinical disease is poorly understood. Specifically, the few studies performed to date on the frequency of stx_2 variants in human isolates [8, 11, 12, 14, 18, 24] often were done on a relatively small scale, comprised selected groups of individuals (subjects with HUS or diarrhea or who were asymptomatic carriers) and/or of STEC (O157 or non-O157), and examined a limited spectrum of stx_2 variants.

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To better understand the significance of STEC harboring stx_2 variants in human disease, we investigated all stool samples submitted to our laboratory for routine microbiological diagnosis between 1996 and 2000, using a spectrum of polymerase chain reaction (PCR) procedures to detect stx_1, stx_2, and stx_3 variants, including stx_{2c}, stx_{2d}, stx_{2e}, and stx_{2g}. We determined the relative frequency of STEC harboring the stx_2 gene variants and their association with clinical symptoms. We also serotyped the isolates and assessed the presence of the *eae* gene, an important accessory virulence gene of STEC [21, 23]. Furthermore, we investigated whether or not the STEC harboring stx_2 variants expressed Stx and whether or not these toxins could be detected by commercial Stx immunoassays.

Materials and Methods

Stool specimens and isolation of STEC. From January 1996 to December 2000, 5487 stool samples from patients with classic HUS ($n = 510$) and patients with bloody ($n = 67$) or watery ($n = 4910$) diarrhea were investigated for the presence of STEC during routine diagnostic work in the Institute for Hygiene and Microbiology (University of Würzburg, Würzburg, Germany). Screening for and isolation of STEC from stools were performed as follows: 1 g of stool was grown in 10 mL of GN Broth Hajna (Difco Laboratories) for 6 h. *E. coli* O157 was sought in 1 mL of this enrichment culture by use of an immunomagnetic separation (IMS) technique and subsequent culture of magnetically separated organisms on sorbitol MacConkey (SMAC) agar and cefixime-tellurite (CT)–SMAC agar, as described elsewhere [25]. To find non-O157 STEC, 200 μL of the enrichment culture was cultured on SMAC agar. The overnight bacterial growth was harvested into 1 mL of saline, and 10^8 cells were used in the PCR with the primer pairs KS7 and KS8 [26] and LP43 and LP44 [27], which complement the stx_1, stx_2, and stx_3 variant genes, respectively (table 1). This PCR screening also was applied to the IMS-processed cultures from SMAC agar and CT-SMAC agar plates, to look for sorbitol-fermenting STEC O157. The PCRs were performed with the GeneAmp PCR System 9600 (Perkin Elmer–Applied Biosystems) in a volume of 50 μL that contained 5 μL of bacterial suspension (10^8 cells), 200 μM each dNTP, 30 pmol of each primer, 5 μL of 10-fold–concentrated polymerase synthesis buffer, 1.5 mM $MgCl_2$, and 2.0 U of AmpliTaq DNApolymerase (Perkin Elmer–Applied Biosystems). The PCR conditions are shown in table 1. The amplification products were subjected to submarine gel electrophoresis in a 1.5% (wt/vol) agarose gel and were visualized by staining with ethidium bromide. To identify STEC in PCR-positive samples, colony blot hybridization with 100–200 well-separated colonies was performed by use of digoxigenin-labeled stx_1 and stx_2 probes prepared with primer pairs KS7-KS8 and LP43-LP44, respectively, from *E. coli* O157:H7 strain EDL 933 [28], as described by Schmidt et al. [26].

In addition to STEC isolated from patients with HUS or from patients with diarrhea without HUS, 96 STEC strains originating from asymptomatic individuals were included in the present study. These organisms were isolated between 1996 and 2000 in the Governmental Institute of Public Health Lower Saxony (Hannover, Germany) during epidemiological investigations and were submitted to our laboratory for further characterization.

stx genotyping and detection of the eae gene. The *stx* genotypes and the presence of the *eae* gene in STEC isolates were determined by PCRs that used the primers and conditions shown in table 1. The strategy to detect stx_2 variants was as follows: STEC that were positive in the PCR with primer pair LP43 and LP44 (which detects A subunit genes of stx_2 and stx_2 variants; table 1) were subjected to PCR with primer pair GK3 and GK4 [29] (table 1). The GK3-GK4

Table 1. Polymerase chain reaction (PCR) primers and conditions used in the present study.

Primer	Sequence	Target	PCR conditions, °C, s[a]			Length of PCR product, bp	Reference
			Denaturing	Annealing	Extension		
KS7	5′-CCC GGA TCC ATG AAA AAA ACA TTA TTA ATA GC-3′	stx_1	94, 30	52, 60	72, 40	282	[26]
KS8	5′-CCC GAA TTC AGC TAT TCT CAC TCA ACC-3′						
LP43	5′-ATC CTA TTC CCG GGA GTT TAC G-3′	stx_2 and variants[b]	94, 30	57, 60	72, 60	584	[27]
LP44	5′-GCG TCA TCG TAT AGA CAG GAG C-3′						
GK3	5′-ATG AAG AAG ATG TTT ATG-3′	stx_2A, stx_2B	94, 30	52, 60	72, 40	260	[29]
GK4	5′-TCA GTC ATT ATT AAA CTG-3′						
VT2-c	5′-AAG AAG ATA TTT GTA GGG G-3′	stx_2B	94, 30	55, 60	72, 60	256	[8]
VT2-f	5′-TAA ACT GCA CTT CAG CAA AT-3′						
FK1	5′-CCC GGA TCC AAG AAG ATG TTT ATA G-3′	stx_2B	94, 30	55, 60	72, 40	281	[30]
FK2	5′-CCC GAA TTC TCA GTT AAA GTT CAC C-3′						
128-1	5′-AGA TTG CGC GTC ATT CAC TGG TTG-3′	stx_2A	94, 30	57, 60	72, 60	420	[10]
128-2	5′-TAC TTT AAT GGC CGC GCT GTC TCC-3′						
SK1	5′-CCC GAA TTC GGC AGA AGC ATA AGC-3′	*eae*	94, 30	52, 60	72, 60	863	[24]
SK2	5′-CCC GGA TCC GTC TGG CCA GTA TTC G-3′						

NOTE. *stx*, Shiga toxin gene.

[a] All PCRs included 30 cycles, followed by a final extension step of 5 min at 72°C.

[b] stx_{2A}, stx_{2B}, and stx_{2vhA} (10).

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GK4	5′-TCA GTC ATT ATT AAA CTG-3′						
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VT2-f	5′-TAA ACT GCA CTT CAG CAA AT-3′						
PK1	5′-CCC GGA TCC AAG AAG ATG TTT ATA G-3′	stx_2B	94, 30	55, 60	72, 40	280	[30]
PK2	5′-CCC GAA TTC TCA GTT AAA CTT CAC C-3′						
128-1	5′-AGA TTG GGC GTC ATT CAC TGG TTG-3′	eae_β	94, 30	57, 60	72, 60	428	[10]
128-2	5′-TAC TTT AAT GGC CGC CCT GTC TCC-3′						
SK1	5′-CCC GAA TTC GGC ACA AGC ATA AGC-3′	eae	94, 30	52, 60	72, 60	663	[26]
SK2	5′-CCC GGA TCC GTC TCG DCA GTA TTC G-3′						

NOTE. *stx*, Shiga toxin gene.

[a] All PCRs included 30 cycles, followed by a final extension step of 5 min at 72°C.

[b] stx_2A, $stx_{2c}A$, and $stx_{2d}A$ [10].

amplification products were digested with restriction endonucleases *Hae* III and *Fok*I (Boehringer Mannheim GmbH), to differentiate B subunit genes of stx_2 and stx_{2c}, as described by Rüssmann et al. [11]. Isolates from which amplification products were not elicited with primers GK3 and GK4 were tested for the stx_{2c} gene by use of the primer pair FK1 and FK2 [30] (table 1). The isolates that did not contain stx_2, but reacted with primers LP43 and LP44, which suggests the presence of another stx_2 allele, were tested for the presence of the stx_{2d} gene with the primer pair VT2-cm and VT2-f [8] (table 1). stx_{2d} is defined in the present study as a stx_2 variant amplified with primers VT2-cm and VT2-f [8] and does not refer to the intestinal mucus–activated Stx2d toxin subtype (encoded by stx_2 variants that have been classified as stx_{2c} [31, 32]), as defined by Melton-Celsa et al. [33]. In addition, all STEC isolated during the study period were investigated for the stx_{2g} gene with the primer pair 128-1 and 128-2 [10] (table 1).

E. coli O157:H7 strain EDL 933 [26, 28] was used as the positive control in the PCRs for the detection of stx_1, stx_2, and *eae* genes. Strains E32511 (O157:H⁻; stx_{2c}) [34], EH250 (ONT:H12; stx_{2d}) [8], ED-53 (O101:H⁻; stx_{2c}) [35], and T4/97 (O128:H2; stx_{2g}) [10] were used as positive controls in PCRs for the detection of the respective stx_2 variants.

Phenotypic methods. STEC isolates were serotyped according to the method of Bockemühl et al. [36] with the use of antisera against *E. coli* O antigens 1–173 and *E. coli* H antigens 1–56. Fermentation of sorbitol was detected on SMAC agar after overnight incubation [37]. Stx production was tested by use of the Vero cell cytotoxicity assay [38] and 2 different commercial Stx assays, including an Stx EIA (Ridascreen Verotoxin; R-Biopharm, GmbH) and a latex agglutination assay (VTEC-RPLA [Verotoxin-producing *E. coli* reverse passive latex agglutination]; Denka Seiken). The Vero cell cytotoxicity assay was performed as described elsewhere [38]. The Stx EIA was performed according to the manufacturer's instructions, with bacterial cultures enriched overnight in a medium that contained mitomycin C (EHEC Direct Medium; Helpha). The latex agglutination assay was performed as described by Karmali et al. [39], with supernatants of overnight cultures in Tryptic Soy Broth (Difco Laboratories) that were diluted from 1:2 to 1:128. The agglutination was examined visually after 20–24 h of incubation, and the toxin titers were expressed as the reciprocals of the highest dilutions that caused agglutination.

Case definition. Patients diagnosed as having diarrhea had ≥3 semisolid or liquid stools per day. Bloody diarrhea was defined as diarrhea in which visible blood was noted in the stool. HUS was defined as hemolytic anemia (hematocrit <30%, with evidence of the destruction of erythrocytes on a peripheral blood smear), thrombocytopenia (platelet count <150,000 cells/mm³), and renal insufficiency (a serum creatinine concentration that exceeded the upper limit of the normal range for age) preceded by diarrhea [40]. Asymptomatic carriers were apparently healthy individuals without diarrhea.

Statistical analysis. Differences between groups were assessed by use of the χ^2 test and Yates's corrected χ^2 test for small numbers [41]. Epi-Info software (version 6.04b, Centers for Disease Control and Prevention and World Health Organization) was used to perform calculations. $P < .05$ was considered to be statistically significant.

Results

Isolation of STEC and serotyping. Between January 1996 and December 2000, stool samples from 549 (10.0%) of the 5487 individuals investigated in the Institute for Hygiene and Microbiology (University of Würzburg, Würzburg, Germany) were positive for the stx_1 and/or stx_2 gene by PCR screening with primers KS7-KS8 and LP43-LP44. STEC strains could be isolated from 530 (96.5%) of 549 PCR-positive stool samples. The proportion of STEC colonies identified by colony blot hybridization with the stx_1 and stx_2 probes ranged from 0.5% to 65.0% in individual stool samples. One STEC isolate per patient was chosen randomly for further analysis. In total, 268 STEC isolates were obtained from 510 patients with HUS, and 262 isolates were obtained from 4977 patients with diarrhea who did not develop HUS. Fourteen of 262 isolates were from patients with bloody diarrhea, and 248 isolates were from 4910 patients with watery diarrhea without blood. The STEC isolation rates from patients with HUS, bloody diarrhea, and watery diarrhea were 52.5%, 20.9%, and 5.1%, respectively. Nine of 530 STEC isolates originated from 3 clusters of STEC O157 infection in families, and 11 strains were from 4 clusters of STEC O26-associated diarrhea and HUS. All the remaining 510 STEC isolates were from apparently sporadic cases of infection, without obvious geographic or temporal linkage. Among 96 STEC isolates from asymptomatic individuals provided by the Governmental Institute of Public Health Lower Saxony (Hannover, Germany), 7 originated from family members of 4 patients with HUS, and 3 were from siblings of 3 patients with diarrhea. The other 86 STEC isolates had no obvious epidemiological associations with the investigated patients. They were isolated from healthy children and adults in kindergartens, schools, factories, and community during environmental investigations. Three of these 86 STEC isolates were isolated from workers of the same meat processing plant; the other 83 were, to our knowledge, epidemiologically unrelated. Three hundred and nine of the 626 STEC isolates investigated in the present study have been described elsewhere [16, 21, 38, 42–44]. The ages of subjects from whom STEC were isolated ranged from 2 months to 73.5 years (median, 4.3 years) for patients with HUS, from 1 month to 86 years (median, 9 years) for patients with diarrhea who did not develop HUS, and from 37 months to 82 years (median, 10.5 years) for asymptomatic individuals.

One hundred and thirty-nine (51.9%) of 268 STEC isolates from patients with HUS were classic non–sorbitol-fermenting *E. coli* O157:H7 or O157:H⁻ (table 2); the remainder were sorbitol-fermenting STEC O157:H⁻ and non-O157 STEC isolates, which most frequently belonged to serogroup O26 (table 2). In patients with diarrhea, non-O157 STEC isolates accounted for most of the isolates, and approximately half belonged to a broad spectrum of serotypes that were not detected in patients with HUS (table 2). The 14 STEC isolates from patients with bloody diarrhea belonged to serotypes O157:H7 (11 isolates), O103:

H^- (2 isolates), and O145:H^- (1 isolate). STEC isolates from asymptomatic carriers belonged mostly (78.1%) to non-O157 serotypes that were not associated with HUS, but some of these isolates had serotypes that were identical to those of STEC isolates from patients with diarrhea (table 2). Three hundred and thirty-four (96.0%) of 348 non-O157 STEC isolates isolated during the study fermented sorbitol.

stx genotypes of STEC and the frequency of isolates harboring stx_2 variants. The 626 STEC isolates belonged to 10 different *stx* genotypes (table 3). stx_2 variants included stx_{2c}, stx_{2d}, and stx_{2e} and were detected in 226 (36.1%) of 626 isolates (table 3). stx_{2c} was the most frequent stx_2 variant. It was found in 148 (23.6%) of 626 STEC isolates but usually in combination with other *stx* genes, most frequently with stx_1 (table 3). Specifically, 28 (4.5%) of 626 STEC isolates contained stx_{2c} as the sole *stx* gene (table 3). stx_{2d}, either alone or together with stx_1, was identified in 62 (9.9%) of 626 STEC isolates, and stx_{2e}, always as a single *stx* gene, was identified in 16 (2.6%) (table 3). None of the STEC isolates in the present study contained stx_{2f}.

Serotypes and frequency of eae in STEC harboring stx_{2c}, stx_{2d}, or stx_{2e} alleles. All STEC isolates that contained stx_{2c} only, stx_{2d} or stx_{2e} were serotyped and investigated for the presence of *eae* (table 4). Although 19 (67.9%) of the 28 isolates of the stx_{2c} genotype were STEC O157:H7 or O157:H^-, none of the 78 STEC isolates that contained stx_{2d} or stx_{2e} alleles was *E. coli* O157 (table 4). Also, none of the latter 78 isolates belonged to any of the major non-O157 STEC serogroups, including O26, O103, O111, and O145. Instead, these 78 isolates belonged to 15 other non-O157 serogroups (table 4). Thirteen (35.1%) of 37 isolates of the *stx*, and stx_{2c} genotype clustered in serogroup O128 (table 4). Five of 7 isolates of the stx_{2c} genotype whose O antigens were typeable belonged to the serogroup O60 and the other 2 to the serogroup O8 (table 4). However, 10 (62.5%) of 16 isolates harboring stx_{2e} and 16 (25.8%) of 62 isolates that contained stx_{2d} possessed O or H antigens that were not typeable with the O and H antisera used (table 4); such isolates might represent new serotypes. Moreover, 6 of the 62 isolates that contained stx_{2d} were able to be autoagglutinized (table 4).

eae was present in 22 (78.6%) of 28 STEC isolates of the stx_{2c} genotype, including each of the O157:H7/H^- STEC isolates that harbor this allele (table 4). However, *eae* was absent from each of the 78 STEC isolates that contained stx_{2d} or stx_{2e} alleles ($P < .000001$; table 4). The absence of *eae* from each of the 78 STEC isolates that harbored stx_{2d} or stx_{2e} was also highly significant when these isolates were compared with the 193 STEC isolates of the stx_2 genotype, among which 97.9% were *eae* positive ($P < .000001$; table 4), and with the 87 non-O157 STEC of the stx_2 genotype (95.4% *eae* positive; $P < .000001$; table 4). The association was still significant when the 78 STEC harboring stx_{2d} or stx_{2e} were compared with the 9 non-O157 STEC of the stx_{2c} genotype (33.3% *eae* positive; $P = .0003$; table 4). In the group of the 28 STEC isolates of the stx_{2c} genotype, the frequency of *eae* among O157 isolates (100%) was significantly

Table 2. Serotypes of 626 Shiga toxin–producing *Escherichia coli* (STEC) isolated from patients with hemolytic-uremic syndrome (HUS), patients with diarrhea, and asymptomatic individuals.

Serotype	HUS	Diarrhea without HUS	Asymptomatic	Total isolates
O157:H7/H^-, NSF	139 (51.9)	77 (20.4)	8 (8.3)	224 (35.5)
O157:H^-, SF	39 (14.5)	16 (4.1)	1 (1.0)	56 (8.9)
O26:H11/H^-	35 (13.0)	28 (10.7)	4 (4.2)	67 (10.7)
O145:H^-	19 (7.1)	15 (5.7)	3 (3.1)	37 (5.9)
O103:H2/H18/H^-	9 (3.4)	25 (9.5)	5 (5.2)	39 (6.2)
O111:H^-	8 (3.0)	13 (5.0)	2 (2.1)	23 (3.7)
Others	19 (7.1)[a]	88 (33.6)[b]	73 (78.1)[c]	182 (29.1)
Total	268 (100)	262 (100)	96 (100)	626 (100)

NOTE. Data are no. (%) of isolates. H^-, nonmotile; NHT, H antigen nontypeable; NSF, non–sorbitol fermenting; ONT, O antigen nontypeable; Orough, autoagglutinable strains; SF, sorbitol fermenting.

[a] O4:H^-, O8:H2, O8:H19, O11:H2, O25:H^-, O55:H6, O70:H25, O77:H^-, O84:H^-, O92:H33, O92:HNT, O112ac:H^-, O118:H^-, O119:H2, O121:H10, O165:H^-, ONT:H^-, Orough:H11, and Orough:H25.

[b] O3:H2, O3:H10, O6:H^- (2 strains), O8:H4, O8:H^- (2 strains), O8:HNT, O16:H32, O16:H48, O22:H8, O23:H10, O25:H^-, O30:HNT, O31:H^-, O40:H6, O40:H^-, O60:H2 (2 strains), O60:H19, O97:H^- (2 strains), O62:H^- (2 strains), O69:H8, O70:H^-, O74:H^-, O75:H8, O75:H21, O75:H^-, O78:H^- (3 strains), O84:H6, O91:H^- (2 strains), O96:H^-, O113:H4, O113:H6, O113:H18, O115:H^- (3 strains), O120:HNT, O121:H^-, O128:H2 (5 strains), O128:H^- (4 strains), O128:HNT, O129:H^-, O146:H21, O146:HNT (3 strains), O152:H4, ONT:H4, ONT:H8 (6 strains), ONT:H16, ONT:H18, ONT:H^- (11 strains), Orough:H19, Orough:H^- (6 strains), and Orough:HNT.

[c] O1:H^-, O3:H^- (2 strains), O6:HNT (3 strains), O8:H^- (2 strains), O16:H^- (2 strains), O21:H^-, O22:H8, O25:HNT (2 strains), O31:H^-, O35:HNT, O40:H8, O60:H2 (3 strains), O60:H^- (2 strains), O62:H^- (3 strains), O71:H^-, O73:HNT, O75:H21 (2 strains), O75:H33, O80:H^-, O82:H^-, O91:H^- (3 strains), O95:H^-, O99:HNT, O104:H16, O113:H4, O115:H^-, O116:H25, O120:H^-, O122:H^-, O123:H^- (2 strains), O128:H2 (5 strains), O128:H^- (3 strains), O151:H^-, O162:H^-, O174:H^-, ONT:H2 (2 strains), ONT:H6 (2 strains), ONT:H8 (3 strains), ONT:H10, ONT:H14 (2 strains), ONT:H^- (7 strains), Orough:H5, and Orough:H^-.

higher than that among non-O157 isolates (33.3%; $P = .0004$; table 4). In total, the frequency of *eae* among the 28 STEC isolates of the stx_{2c} genotype (78.6%) was significantly lower than that among the 193 STEC isolates of the stx_2 genotype (97.9%; $P = .00004$; table 4).

Association between STEC harboring stx_2 variants and clinical manifestations of the infection. To investigate the association between stx_2 variants and clinical manifestations of the infection, we compared the relative frequency of STEC isolates that possessed stx_{2c} only, stx_{2d}, or stx_{2e} among STEC isolates from patients with HUS, STEC isolates from patients with diarrhea who did not develop HUS, and STEC isolates from asymptomatic individuals (table 3). Moreover, we determined the distribution of STEC harboring each of the respective stx_2 gene variants between patients with HUS and those individuals who were infected but did not develop HUS (i.e., patients with diarrhea and asymptomatic subjects; table 5), to assess the potential of such infections to progress to HUS.

stx_{2c} was the only stx_2 variant associated with HUS. However, the proportion of STEC isolates that possessed the stx_{2c} genotype was similar among isolates from patients with HUS (3.7%), isolates from patients with diarrhea who did not develop HUS (5.0%), and isolates from asymptomatic individuals (5.2%) (table 3). Moreover, no significant difference was found in the distribution of the 28 STEC isolates of the stx_{2c} genotype between patients with HUS (10 [35.7%] of 28) and those who did not develop HUS (patients with diarrhea or asymptomatic subjects; 18 [64.3%] of 28; $P = .21$) (table 5). This lack of association applied to the 19 isolates of O157 serogroup (table 4), 5 of which were associated with HUS, and to the 9 non-O157 isolates of the stx_{2c} genotype (table 4), 5 of which were associated with HUS (data not shown). In addition, the proportions of STEC isolates of the stx_{2c} genotype that contained the *eae* gene were comparable among isolates from patients with HUS (7/10) and among isolates from individuals who did not develop HUS (15/18; data not shown). Of 13 patients with diarrhea from whom STEC of the stx_{2c} genotype were isolated (table 3), the stool sample of 1 patient was bloody; the infecting strain belonged to the serotype O157:H7. The other 12 patients, 8 of whom were infected with STEC O157:H7/H$^-$ and 4 with non-O157 STEC, had diarrhea without visible blood.

In contrast to stx_{2c}, the stx_{2d} gene was identified in none of 268 STEC isolates from patients with HUS but was present in 41 (15.6%) of 262 STEC isolated from patients with diarrhea ($P < .000001$) and in 21 (21.9%) of 96 STEC isolated from asymptomatic individuals ($P < .000001$) (table 3). The signifi-

Table 3. Shiga toxin gene (*stx*) genotypes of 626 Shiga toxin–producing *Escherichia coli* (STEC) and their relative frequency among isolates from patients with hemolytic-uremic syndrome (HUS), patients with diarrhea, and asymptomatic individuals.

stx genotype	HUS	Diarrhea without HUS	Asymptomatic[a]	Total isolates
stx_1	10 (3.7)	111 (42.4)	45 (46.8)	166 (26.5)
stx_2	147 (54.9)[b]	41 (15.7)[b]	5 (5.2)[b]	193 (30.8)
stx_2 + stx_1	26 (9.7)	9 (3.4)	6 (6.3)	41 (6.5)
stx_{2c}	10 (3.7)[c]	13 (5.0)[c]	5 (5.2)[c]	28 (4.5)
stx_2 + stx_{2c}	6 (2.2)	14 (5.3)	6 (6.3)	26 (4.2)
stx_2 + stx_{2d}	68 (25.4)	17 (6.5)	3 (3.1)	88 (14.0)
stx_2 + stx_2 + stx_{2c}	1 (0.4)	4 (1.5)	1 (1.0)	6 (1.0)
stx_{2d}	0 (0)[d,e]	18 (6.7)[d,e]	10 (10.4)[d,e]	28 (4.5)
stx_1 + stx_{2d}	0 (0)[d,f]	26 (9.9)[d,f]	11 (11.5)[d,f]	37 (5.9)
stx_{2e}	0 (0)[g]	17 (6.5)[g]	4 (4.2)[g]	16 (2.6)
Total	268 (100)	262 (100)	96 (100)	626 (100)

NOTE. Data are no. (%) of isolates. The χ^2 test and Yates's corrected (YC) χ^2 test were used for calculations. $P < .05$ was considered to be statistically significant.

[a] Three, 2, 2, and 3 of the isolates of genotypes stx_1, stx_2, stx_2 + stx_1, and stx_1 + stx_{2c}, respectively, originated from asymptomatic contacts of 4 patients with HUS and 3 patients with diarrhea. Three of 10 STEC of the stx_{2c} genotype were isolated from workers of the same meat-processing plant.

[b] The relative frequency of STEC of the stx_2 genotype among STEC from patients with HUS vs. diarrhea, $P < .000001$, χ^2; 1 df, 88.95; HUS vs. asymptomatic, $P < .000001$, χ^2; 1 df, 71.62; and diarrhea vs. asymptomatic, $P = .019$, χ^2; 1 df, 0.84.

[c] The relative frequency of STEC of the stx_{2c} genotype among STEC from patients with HUS vs. diarrhea, $P = .49$, χ^2; 1 df, 0.48; HUS vs. asymptomatic, $P = .74$; YC χ^2, 0.11; and diarrhea vs. asymptomatic, $P = .86$; YC χ^2, 0.03.

[d] The relative frequency of all STEC with stx_{2d} (stx_{2d}, stx_1 + stx_{2d}) among STEC from patients with HUS vs. diarrhea, $P < .000001$, χ^2; 1 df, 46.84; HUS vs. asymptomatic, $P < .000001$, χ^2; 1 df, 31.52; and diarrhea vs. asymptomatic, $P = .17$, χ^2; 1 df, 1.90.

[e] The relative frequency of STEC of the stx_{2d} genotype among STEC from patients with HUS vs. diarrhea, $P = .00007$, χ^2; 1 df, 15.79; HUS vs. asymptomatic, $P = .000001$; YC χ^2, 24.94; and diarrhea vs. asymptomatic, $P = .12$, χ^2; 1 df, 2.36.

[f] The relative frequency of STEC harboring stx_1 + stx_{2d} among STEC from patients with HUS vs. diarrhea, $P < .000001$, χ^2; 1 df, 27.97; HUS vs. asymptomatic, $P < .000001$; YC χ^2, 27.88; and diarrhea vs. asymptomatic, $P = .67$, χ^2; 1 df, 0.18.

[g] The relative frequency of STEC of the stx_{2e} genotype among STEC from patients with HUS vs. diarrhea, $P = .0004$, χ^2; 1 df, 12.36; HUS vs. asymptomatic, $P = .005$; YC χ^2, 7.78; and diarrhea vs. asymptomatic, $P = .90$; YC χ^2, 0.01.

Table 4. Serotypes and frequency of the gene encoding intimin (*eae*) in Shiga toxin–producing *Escherichia coli* (STEC) harboring Shiga toxin gene (*stx*) stx_{2c} only, stx_{2c} or stx_{1c} compared with STEC of the stx_2 genotype.

	O157 serogroup		Non-O157 serogroups		Total isolates	
stx genotype	No. of isolates	No. (%) *eae* positive	No. of isolates	No. (%) *eae* positive[a]	No. of isolates	No. (%) *eae* positive[b]
stx_{2c}	19	19 (100.0)[c]	9[d]	3 (33.3)[e]	28	22 (78.6)
stx_{2c}	0	NA	25[e]	0	25	0
$stx_2 + stx_{1c}$	0	NA	37[f]	0	37	0
stx_{2c}	0	NA	16[g]	0	16	0
stx_2	106	106 (100.0)	87[h]	83 (95.4)	193	189 (97.9)

NOTE. H⁻, nonmotile; HNT, H antigen nontypeable; NA, not applicable; ONT, O antigen nontypeable; Orough, nonagglutinable strains. The χ^2 test and Yates's corrected (YC) χ^2 test were used for calculations. $P < .05$ was considered to be statistically significant.

[a] STEC containing stx_{2c} ($stx_{2c}/stx_1 + stx_{2c}$) or stx_{2c} vs. STEC containing stx_{2c}, $P < .000001$, χ^2; 1 *df*; 149.74. STEC containing stx_{2c} ($stx_{2c}/stx_1 + stx_{2c}$) or stx_{2c} vs. STEC containing stx_{2c}, $P = .0003$; YC χ^2, 13.16.

[b] STEC containing stx_{2c} ($stx_{2c}/stx_1 + stx_{2c}$) or stx_{2c} vs. STEC containing stx_{2c}, $P < .000001$, χ^2; 1 *df*; 77.34. STEC containing stx_{2c} ($stx_{2c}/stx_1 + stx_{2c}$) or stx_{2c} vs. STEC containing stx_{2c}, $P < .000001$, χ^2; 1 *df*; 252.44. STEC containing stx_{2c} vs. STEC containing stx_{2c}, $P = .00004$; YC χ^2, 16.96.

[c] $P = .0006$; YC χ^2, 12.40.

[d] Serotypes O23:H⁻, O39:HNT, O60:H19, O77:H⁻, O92:HNT, O120:HNT, O145:H⁻ (2 strains), and Orough:H⁻.

[e] Serotypes O8:H⁻ (2 strains), O16:H32, O16:H48, O40:H⁻, O40:H8, O40:H8, O62:H⁻ (2 strains), O91:H⁻ (3 strains), O112:H4, O123:H⁻ (2 strains), O146:HNT (2 strains), ONT:H8 (3 strains), ONT:H⁻ (2 strains), Orough:H⁻ (2 strains), and Orough:HNT.

[f] Serotypes O12:H8 (2 strains), O62:H⁻, O70:H⁻, O75:H8, O75:H21, O91:H⁻ (2 strains), O96:H⁻ (2 strains), O113:H⁻ (2 strains), O128:H2 (8 strains), O128:H⁻ (4 strains), O128:HNT, O146:H21, O174:H⁻, ONT:H8 (3 strains), ONT:H⁻ (4 strains), Orough:H19, and Orough:H⁻ (2 strains).

[g] Serotypes O4:HNT, O8:H⁻, O60:H2, O60:H⁻ (4 strains), and ONT:H⁻ (9 strains).

[h] Serotypes O25:H11 (39 strains), O145:H⁻ (23 strains), O103:H⁻ (5 strains), O63:H⁻ (3 strains), O8:H2 (3 strains), O8:H⁻ (3 strains), O70:H35 (2 strains), O11:H2, O23:H19, O41:H⁻, O55:H6, O113:H4, O121:H10, O121:H19, O121:H⁻, and Orough:H⁻.

costly higher frequency among isolates from patients with diarrhea and among isolates from asymptomatic individuals, compared with that among isolates from patients with HUS, was observed for the 25 STEC isolates that harbored stx_{2c} as the sole *stx* gene (5.7%, 10.4%, and 0% of isolates from subjects with diarrhea, without symptoms, and with HUS, respectively: $P = .00007$ and $P = .000001$ for isolates from patients with diarrhea and for isolates from asymptomatic subjects, respectively, vs. isolates from patients with HUS; table 3) and for the 37 STEC isolates that contained stx_{2c} together with stx_1 (9.9%, 11.5%, and 0% of isolates from patients with diarrhea, asymptomatic subjects, and patients with HUS, respectively; $P < .000001$ for both isolates from patients with diarrhea and isolates from asymptomatic subjects vs. isolates from patients with HUS; table 3). However, no significant difference was observed in the relative frequency of STEC harboring stx_{2c} among STEC isolates from patients with diarrhea (15.6%) and among STEC isolates from asymptomatic individuals (21.9%; $P = .17$; table 3). The lack of significant association with diarrhea versus asymptomatic infection applied to the 25 STEC isolates containing stx_{2c} only and to the 37 STEC isolates containing stx_{2c} and stx_1 (table 3). All patients with diarrhea from whom STEC isolates containing stx_{2c} were isolated had diarrhea without visible blood. The highly significant association with uncomplicated infection (including nonbloody diarrhea or asymptomatic infection) versus HUS was confirmed for both the respective groups of the stx_{2c}-containing STEC isolates on the basis of the distributions of such isolates between patients with HUS (0%) and those infected individuals who did not develop HUS (100%; $P < .000001$; table 5).

Similar to stx_{2c}, stx_{2c} was absent from all 268 STEC isolated from patients with HUS but was present in 12 (4.6%) of 262 STEC isolates from patients with diarrhea ($P = .0004$) and in 4 (4.2%) of 96 STEC isolated from asymptomatic individuals ($P = .005$; table 3). Also, as in STEC harboring stx_{2c}, the difference in the relative frequency of stx_{2c}-harboring STEC among STEC from patients with diarrhea (4.6%) and among STEC from asymptomatic subjects (4.2%) was not statistically significant ($P = .90$; table 3). All 12 patients infected with STEC harboring stx_{2c} had nonbloody diarrhea. The association of STEC harboring stx_{2c} with uncomplicated infection that did not progress to HUS was highly significant when the distribution of the 16 stx_{2c}-harboring STEC between patients with HUS (0/16) and those individuals who did not develop HUS (16/16) was compared ($P < .000001$; table 5).

In contrast to STEC harboring the stx_2 variants, STEC of the stx_2 genotype were significantly associated with HUS. The relative frequency of such strains among STEC from patients with HUS was 54.9%, whereas that among isolates from patients with diarrhea who did not develop HUS was 15.7% ($P < .000001$), and that among asymptomatic individuals was 5.2% ($P < .000001$; table 3). Moreover, 147 (76.2%) of 193 STEC isolates of the stx_2 genotype originated from patients with HUS, but only 46 (23.8%) of these strains were isolated from subjects in which the infection did not progress to HUS ($P < .000001$; table 5). Also, in contrast to all stx_2 variants that were not significantly associated with diarrhea versus asymptomatic infection (table 3), the relative frequency of STEC of the stx_2 genotype among isolates from patients with diarrhea (15.7%) was significantly higher than that among STEC from

Table 5. Association of Shiga toxin–producing *Escherichia coli* (STEC) harboring Shiga toxin 2 gene (*stx*$_2$) variants with clinical manifestations of the infection and with age of patients, compared with STEC of the *stx*$_2$ genotype.

stx genotype	Total no. of isolates	Isolates from patients with HUS, no. (%)[a]	Isolates from patients without HUS (with D or A), no. (%)	*P*	χ^2, 1 *df*	Age of patients, median years (range)	Patients <5 years old, no. (%)[b]	Patients >18 years old, no. (%)[c]
stx$_{2c}$	28	10 (35.7)	18 (64.3)	.21	1.54	3 (9 mo–64 y)	14 (50.0)	6 (21.4)
stx$_{2d}$	25	0 (0)	25 (100.0)	<.000001	50.00	38.5 (9 mo–86 y)[d]	4 (16.0)[e]	16 (64.0)[f]
stx$_2$ + *stx*$_{2c}$	37	0 (0)	37 (100.0)	<.000001	74.00	28 (4 mo–66 y)[g]	5 (13.5)[h]	25 (67.6)[i]
stx$_{2e}$	16	0 (0)	16 (100.0)	<.000001	32.00	19.5 (14 mo–59 y)	4 (25.0)[j]	9 (56.3)[k]
stx$_2$	193	147 (76.2)	46 (23.8)	<.000001	105.71	3.5 (2 mo–30 y)	169 (87.6)	6 (3.1)

NOTE. A, asymptomatic; D, diarrhea; HUS, hemolytic-uremic syndrome. The χ^2 test and Yates's corrected (YC) χ^2 test were used for calculations. $P < .05$ was considered to be statistically significant.

[a] Relative risk of HUS development was as follows: *stx*$_2$ vs. *stx*$_{2c}$, $P = .0001$), χ^2; 1 *df*, 19.65. *stx*$_{2c}$ vs. *stx*$_{2c}$ (*stx*$_{2c}$/*stx*$_{2c}$ + *stx*$_{2d}$), $P = .000004$; YC χ^2, 21.43. *stx*$_{2c}$ vs. *stx*$_{2e}$, $P = .019$; YC χ^2, 5.50).

[b] Proportions of patients <5 years old among patients infected with STEC containing *stx*$_{2c}$ (*stx*$_{2c}$/*stx*$_{2c}$ + *stx*$_{2d}$) vs. *stx*$_{2d}$ vs. *stx*$_2$, $P = .0005$, χ^2; 1 *df*, 12.06; *stx*$_2$ vs. *stx*$_{2c}$, $P = .000005$; YC χ^2, 21.67; and *stx*$_2$ vs. *stx*$_{2c}$ (*stx*$_{2c}$/*stx*$_{2c}$ + *stx*$_{2d}$) or *stx*$_{2c}$, $P < .000001$, χ^2; 1 *df*, 126.60.

[c] Proportions of patients >18 years old among patients infected with STEC containing *stx*$_{2c}$ (*stx*$_{2c}$/*stx*$_2$ + *stx*$_{2d}$) or *stx*$_{2d}$ vs. *stx*$_{2c}$, $P = .000$), χ^2; 1 *df*, 15.06; *stx*$_2$ vs. *stx*$_{2c}$, $P = .0004$; YC χ^2, 12.61; and *stx*$_2$ vs. *stx*$_{2c}$ (*stx*$_{2c}$/*stx*$_{2c}$ + *stx*$_{2d}$) or *stx*$_{2c}$, $P < .000001$, χ^2; 1 *df*, 126.06.

[d] Median age of all 62 patients infected with STEC possessing *stx*$_{2c}$ (*stx*$_{2c}$/*stx*$_{2c}$ + *stx*$_{2d}$) was 31.5 years.

[e] The proportion of patients <5 years old among all 78 patients infected with STEC harboring *stx*$_{2d}$ (*stx*$_{2d}$/*stx*$_{2c}$ + *stx*$_{2d}$) or *stx*$_{2d}$ was 16.7%.

[f] The proportion of patients >18 years old among all 78 patients infected with STEC harboring *stx*$_{2d}$ (*stx*$_{2d}$/*stx*$_{2c}$ + *stx*$_{2d}$) or *stx*$_{2d}$ was 64.1%.

asymptomatic individuals (5.2%; $P = .009$; table 3). Seven of 41 patients with diarrhea from which STEC isolates of the *stx*$_2$ genotype were recovered had stools with visible blood.

When we compared the relative risk of infections with STEC harboring *stx*$_2$ and various *stx*$_2$ variants to progress to HUS, we found that the proportion of patients that developed HUS after infection with STEC of the *stx*$_2$ genotype (76.2%; table 5) was significantly higher than the proportion of patients who developed HUS after infection with STEC of the *stx*$_{2c}$ genotype (35.7%; $P = .00001$; table 5). Similarly, the proportion of patients who developed HUS after infection with STEC of the *stx*$_{2c}$ genotype (35.7%; table 5) was significantly higher than the proportion of those who developed HUS after infection with STEC harboring *stx*$_{2d}$ (0%; $P = .000004$; table 5) or *stx*$_{2e}$ (0%; $P = .019$; table 5).

Association between infections with STEC harboring stx$_2$ *variants and the age of patients.* To determine whether infections with STEC harboring different *stx*$_2$ variants were associated with particular age groups, we compared the ages of patients infected with STEC with the *stx*$_{2c}$, *stx*$_{2d}$, or *stx*$_{2e}$ alleles (table 5). As indicated by median ages of patients and by proportions of patients of <5 and >18 years old, patients infected with STEC containing *stx*$_{2d}$ or *stx*$_{2e}$ alleles were substantially older than patients infected with STEC of the *stx*$_2$ genotype (table 5). Only 13 (16.7%) of 78 patients infected with STEC with *stx*$_{2d}$ or *stx*$_{2e}$ alleles were <5 years old, whereas 14 (50.0%) of 28 patients infected with STEC possessing the *stx*$_{2c}$ genotype belonged to this age group ($P = .0005$; table 5). Moreover, 50 (64.1%) of 78 patients infected with STEC harboring *stx*$_{2d}$ or *stx*$_{2e}$ were >18 years old, whereas only 6 (21.4%) of 28 patients infected with STEC of the *stx*$_{2c}$ genotype were adults ($P = .0001$; table 5). In comparison, the proportion of adults among patients infected with STEC of the *stx*$_2$ genotype (3.1%; table 5) was significantly lower than that among patients infected with STEC of the *stx*$_{2c}$ genotype (21.4%; $P = .0004$) and that among patients infected with STEC harboring *stx*$_{2d}$ or *stx*$_{2e}$ (64.1%; $P < .000001$; table 5). Also, the proportion of patients <5 years old among patients infected with STEC of the *stx*$_2$ genotype (87.6%; table 5) was significantly higher than that among patients infected with STEC of the *stx*$_{2c}$ genotype (50.0%; $P = .000003$) and that among patients infected with STEC harboring *stx*$_{2d}$ or *stx*$_{2e}$ (16.7%; $P < .000001$; table 5).

Toxin production by STEC possessing stx$_2$ *variants and detection of Stx2 variants by commercial immunoassays.* To investigate whether the STEC harboring various *stx*$_2$ variants produced Stx and whether these toxins were detectable by commercially available Stx assays, culture supernatants of the 50 STEC that contained *stx*$_{2c}$, *stx*$_{2d}$, or *stx*$_{2e}$ as the sole *stx* genes and were isolated from patients with diarrhea or HUS (table 3) were tested for the Vero cell cytotoxicity and for their reactivity in the Stx EIA and the latex agglutination assay (table 6). All 23 isolates of the *stx*$_{2c}$ genotype produced the toxin, as detected by the Vero cell assay and by the latex agglutination assay. However, 3 of 23 Stx2c producers tested negative in the Stx EIA (table 6). Among the 15 STEC of the *stx*$_{2d}$ genotype, only 10 produced Stx2d, as demonstrated by their cytotoxicity for Vero cells and by positive results in the Stx EIA and the latex agglutination assay (table 6). The remaining 5 isolates that contained *stx*$_{2d}$ as their sole *stx* gene were not cytotoxic for Vero cells and tested negative in both immunoassays. Stx2c was produced by 11 of 12 isolates harboring *stx*$_{2e}$, but in only 7 of them was the toxin detectable by the Stx EIA and by the latex agglutination assay (table 6). Altogether, 44 (88.0%) of 50 STEC isolates that contained *stx*$_{2c}$, *stx*$_{2d}$, or *stx*$_{2e}$ as the sole *stx* genes produced the respective Stx, and the toxins of 37 (84.1%) and 40 (90.9%) of these 44 strains could be detected by the Stx EIA and by the

Table 6. Toxin production by Shiga toxin–producing *Escherichia coli* (STEC) harboring Shiga toxin gene (*stx*) stx_{2a}, stx_{2c}, or stx_{2d}, and detection of Stx2c, Stx2d, and Stx2c by commercial Shiga toxin (Stx) immunoassays.

stx genotype	Total no. of isolates with genotype[a]	No. of isolates cytotoxic for Vero cells	No. of Vero cell cytotoxic isolates positive in Stx EIA[b]	No. of Vero cell cytotoxic isolates positive in latex agglutination assay[c]	Titers with Stx2 latex reagent, geometric mean (range)[d]
stx_{2c}	23	23	20	23	1:10 (1:4–1:16)
stx_{2d}	15	10	10	10	1:4 (1:2–1:8)
stx_{2c}	12	11	7[e]	7[e]	1:4 (1:2–1:8)
Total	50	44	37	40	1:6 (1:2–1:16)

[a] Only STEC isolated in the Institute for Hygiene and Microbiology (University of Würzburg, Würzburg, Germany) from patients with diarrhea or patients with hemolytic-uremic syndrome were investigated for toxin production.

[b] RIDascreen Verotoxin (R-Biopharm); a mixture of monoclonal antibodies against Stx1 and Stx2 was used as an Stx reagent.

[c] Verotoxin-producing *E. coli* reverse passive latex agglutination (Denka Seiken); latex particles sensitized with polyclonal antibodies against Stx1 and Stx2 were used as Stx1 and Stx2 reagent, respectively.

[d] All 40 isolates were negative (titer < 1:2) with the Stx1 reagent.

[e] The same isolates were positive in both commercial assays.

latex agglutination assay, respectively (table 6). However, the latex agglutination titers of Stx2c, Stx2d, and Stx2e (table 6) were significantly lower than the titers of Stx2 detected by the latex agglutination assay in culture supernatants of STEC of the stx_2 genotype (range, 1:16–1:512; geometric mean, 1:80; data not shown).

Stx production in the 26 STEC isolates that contained stx_{2d} together with stx_1 and originated from patients with diarrhea (table 3) was tested by the Vero cell cytotoxicity assay and by a Stx EIA that detects both Stx1 and Stx2 by using a single Stx reagent (table 6). All 26 isolates were cytotoxic for Vero cells, and all of them tested positive in the Stx EIA (data not shown).

Discussion

The presence of the stx_2 genotype in an infecting STEC isolate has been shown to represent a risk factor for the progression of the infection to microangiopathic sequelae such as HUS [22, 23]. However, the clinical significance of STEC harboring variants of stx_2 is unknown. The present study represents the first attempt to classify STEC harboring the presently known stx_2 variants with respect to their capacity to cause extraintestinal manifestations in humans. We found that STEC harboring stx_{2c} only, stx_{2c} or stx_{2d}, accounted for 14.3% of the 530 STEC isolated from patients during a 5-year period. However, STEC with different stx_2 alleles differed markedly in their association with HUS. Specifically, stx_{2c} was the only stx_2 variant associated with HUS, but the risk of developing HUS after infection with STEC of the stx_{2c} genotype was significantly lower than that after infection with STEC of the stx_2 genotype. In contrast to STEC harboring stx_{2c}, STEC possessing stx_{2d} or stx_{2e} alleles were not associated with HUS in the present study. However, such strains accounted for 20.2% of 262 STEC isolated from patients with diarrhea who did not develop HUS.

The finding of stx_{2d} or stx_{2e} alleles in 26.1% of 96 STEC from asymptomatic individuals of the same population during the same time period has 2 important implications. First, it supports the association of STEC harboring stx_{2d} or stx_{2e} with uncomplicated infection. Second, the comparable frequency of stx_{2d} and stx_{2e} alleles among STEC isolated from patients with diarrhea (20.2%) and among STEC isolated from asymptomatic subjects (26.1%) raises the question about the etiological role of the *E. coli* containing these genes in the diarrhea of the patients from which these strains were isolated. This question cannot be answered in our study, which was focused on the detection of STEC and subtyping of their *stx* genes and did not investigate systematically the presence of other enteric pathogens in the patients. Hence, on the basis of our data, we cannot exclude the possibility that some of the patients from whom STEC harboring stx_{2d} or stx_{2e} were isolated might have been coinfected with obligatory bacterial or viral diarrheagenic pathogens that were, in fact, causative agents of the disease. Ideally, future studies will encompass a broader spectrum of intestinal pathogens to evaluate the etiological role of STEC harboring stx_{2d} or stx_{2e} in diarrhea and thus better understand the pathogenic potential of such strains for humans. The finding of stx_{2d} in 21.9% of STEC from asymptomatic subjects in our study is not surprising, compared with the presence of this stx_2 variant in 65% of 37 STEC isolated from asymptomatic carriers in Switzerland [14]. The presence of stx_{2e} in STEC isolated from asymptomatic individuals has not been reported elsewhere, to our knowledge.

The inference that STEC harboring different stx_2 alleles have different abilities to cause HUS has several diagnostic implications. Most important, it demonstrates the importance of isolating of STEC from stool cultures that are positive for *stx* by PCR screening or for Stx by EIA screening [45]. This should be followed by rapid subtyping of stx_2 genes, because the information about the stx_2 allele, combined with the information about the presence of *eae* in the STEC isolate, has considerable predictive value for the treating physician to assess the risk of HUS development in a patient who presents with STEC infection, if our findings can be validated in prospective studies. In

this respect, *stx* genotyping appears to offer an advantage to serotyping among non-O157 STEC, because STEC harboring stx_{2d} or stx_{2c} belong to a broad spectrum of non-O157 serotypes, but the association between uncomplicated infection that does not show a tendency to progress to HUS and the specific stx_2 alleles persists, independently of serotype. For laboratories that cannot use PCR to subtype *stx* genes in STEC, it is particularly noteworthy that Stx2c, Stx2d, and Stx2e produced by most patients' STEC isolates investigated in this study could be detected by commercially available Stx immunoassays, including EIA and the latex agglutination assay. The inability of the commercial assays to detect Stx2e in 4 producers of this toxin is in agreement with the observation by Beutin et al. [46], who could not detect Stx2e in STEC isolated from pig edema disease using the VTEC-RPLA assay. The reason for the inability of both commercial assays used in this study to detect Stx2e in some of the toxin producers could be either inadequate in vitro production of toxin by such isolates or antigenic differences in the toxin molecules that diminish the recognition of these toxins by antibodies used in the respective commercial immunoassays. In fact, a lower sensitivity of the Stx2 latex agglutination reagent for the detection of Stx2e, compared with that for the detection of Stx2, was observed by Karmali et al. [39] and appears to be the case also for Stx2d and Stx2e, as suggested by the low latex agglutination titers ($\leq$1:8) that we observed in STEC producing these toxins in the present study. From a diagnostic standpoint, such low toxin titers in the latex agglutination assay may suggest production of an Stx2 variant, rather than of the classic Stx2, by the isolate. However, considering the important predictive value of the information about the Stx2 type produced by an STEC isolate, commercial immunoassays that use specific antibodies that would differentiate Stx2 from its variants and identify the respective Stx2 variants should be developed and evaluated. However, it should be remembered that, although toxin detection assays represent valuable adjuncts to culturing, it is crucial to isolate STEC that can be further characterized for clinical, epidemiological, and analytical purposes. Such assays should not be used in lieu of standard microbiologic assessments.

An additional limitation in the use of toxin detection assays is that not all STEC that harbor stx_2 variants produce the respective toxins under laboratory conditions. In our study, the lack of the toxin production was most pronounced in STEC harboring stx_{2d} as the sole *stx* gene. One-third of these isolates (5/15) did not produce Stx2d in vitro, as demonstrated by the absence of the Vero cell cytotoxicity in their culture supernatants. Similarly, lack of toxin production was observed in 1 of 12 STEC that harbored stx_{2c}. Although reasons for the lack of the in vitro toxin production in these STEC are unclear, there are several possible explanations. First, the proteins might not be expressed because mutations in *stx* genes introduce changes in reading frames or stop codons. Second, the genes might not be expressed under the growth conditions used. Third, the toxins were synthesized but were not released from the bacterial cells because of a defect in a toxin export mechanism. However, because the environment in the intestine differs substantially from laboratory conditions, the lack of Stx production in vitro does not exclude the possibility that the toxins were not produced by these STEC in vivo during infection. Investigations are in progress to determine the reason(s) for the inability of the respective isolates to produce the toxins in vitro.

The lack of the association of STEC harboring stx_{2d} or stx_{2c} with HUS, as demonstrated in the present study, may have several explanations. The first is the universal absence of the *eae* gene from all STEC that contain stx_{2d} or stx_{2c}, as observed in this study and reported by Piérard et al. [8, 18]. *eae* is an important accessory virulence gene of STEC isolated from patients with HUS [21], and its presence in STEC O157 and STEC of the 4 major non-O157 serogroups (O26, O103, O111, and O145) has been associated strongly with the ability of such STEC to cause severe human disease, including HUS [23]. Alternatively, Stx2d and Stx2e might be less toxic than Stx2 or Stx2c for humans. Naturally occurring Stx2 variants differ in their virulence in a mouse model [47]. Although not directly comparable to human infection, *E. coli* that expressed Stx2d had lower toxicity than isogenic clones that expressed Stx2c when administered intraperitoneally to mice [47]; oral virulence of 1 of the Stx2d-expressing clones for streptomycin-treated mice was significantly lower than that of both clones that expressed Stx2c [47]. Reduced pathogenicity of STEC harboring stx_{2d} for humans is also suggested by the high frequency of isolation of such STEC from stool samples of asymptomatic individuals [14], an observation that was also confirmed in our study. Another possible reason for the lack of the association of STEC harboring stx_{2d} or stx_{2c} with HUS in our study might be the fact that such STEC infected mostly adults. Only 13 of 78 individuals infected with STEC harboring stx_{2c} or stx_{2d} were $<$5 years old and thus of the age that has been shown to represent a significant risk factor for the development of systemic complications after STEC infection [1]. In this context, it is noteworthy that the previous rare reports on human infections with STEC harboring stx_{2c} mainly have described adult patients [17–19]. The reason why such STEC have a tendency to infect adults rather than young children remains unknown. However, this relationship is obviously complex. For example, stx_1-positive, stx_2-negative STEC are clearly capable of causing HUS [48], and there have been rare reports of STEC harboring stx_{2c} [13, 49] and stx_{2e} [19] being isolated from patients with HUS. In our study, we cannot exclude the possibility that some of the patients with HUS from whom STEC were not recovered, probably because the isolation was not attempted until presentation with HUS, might have been infected with STEC harboring these stx_2 variants.

Similar to the etiological role of STEC containing stx_{2d} or stx_{2c} in diarrhea, the epidemiology of infections caused by such strains is presently unknown. A recent report of the high preva-

lence of stx_{2d} in *E. coli* from the normal intestinal microflora of sheep [13] and the finding of stx_{2d} in selected human isolates that belonged to the same serotypes as ovine stx_{2d}-harboring STEC [13] suggest that sheep could be a reservoir of stx_{2d}-harboring STEC for humans. This would be consistent with our finding of STEC containing stx_{2d} in 3 workers from a meat processing plant. The serogroups identified in stx_{2d}-harboring STEC isolated from patients in this study (O8 and O60) and in other studies (O101 and O9 [17, 19]) have not been associated with pig edema disease [15]. However, the isolation of Stx2e-producing STEC of serogroup O101 from healthy pigs [50] and the finding that such strains demonstrate a high degree of genetic relatedness to a human stx_{2d}-harboring STEC O101 isolate [35] suggest that healthy pigs might be a potential reservoir of stx_{2d}-harboring STEC for humans.

Stx1 is a newly described Stx2 variant that we identified in STEC recovered from feral pigeons [10]. The observation that stx_{2g} of a pigeon isolate is almost identical to a stx_2 variant gene identified previously in an *E. coli* strain isolated in Canada from a patient with diarrhea [20] prompted us to investigate all human STEC in the present study for the presence of stx_{2g}. The uniform absence of stx_{2g} from the 626 STEC suggests that stx_{2g} may have minimal, if any, link to pathogenicity in humans. However, an investigation is in progress to look for stx_{2g} in human *E. coli* isolates that are similar to pigeon stx_{2g}-harboring STEC [10], *eae* positive but negative for other *stx* genes.

In conclusion, STEC possessing different stx_2 variants differ in their capacity to cause HUS. Although infection with STEC of the stx_{2d} genotype can progress to HUS, STEC harboring stx_{2c} or stx_{2g} genes are not associated with HUS but represent a significant part of STEC from patients with uncomplicated diarrhea and are found frequently in asymptomatic STEC carriers. Such isolates lack *eae* and have a tendency to infect adults, rather than young children. The presence of stx_{2c} or stx_{2g} in an STEC isolate, combined with the absence of the *eae* gene, may thus predict mild disease with a minimal risk of HUS development; prospective studies will be needed to test the predictive value and clinical utility of *stx* genotyping efforts.

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Non-O157:H7 Stx2-Producing *Escherichia coli* Strains Associated with Sporadic Cases of Hemolytic-Uremic Syndrome in Adults

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From August 1996 to May 1997, six verotoxin-producing *Escherichia coli* (VTEC) strains were isolated from stool specimens of adults suffering from hemolytic-uremic syndrome (HUS). All the isolates were stx_2 positive and belonged to different serotypes: O6:H4, O91:H10, O91:H21, O rough:H16, OX3:H−, and O nontypeable: H−. The enterohemolysin (Ehly)-encoding genes were detected in two isolates, and none of the isolates harbors the intimin (Eae)-encoding gene. These findings suggest that stx_2-positive non-O157:H7 VTEC is a major cause of HUS in adults and that several sources of pathogens are responsible for local endemic infections.

Hemolytic-uremic syndrome (HUS) is characterized by acute hemolytic anemia, thrombocytopenia, and acute renal failure. In some cases, these three clinical features are associated with neurological manifestations and fever. The association between HUS and verotoxin-producing *Escherichia coli* (VTEC) infection is now well established, and usually prodromic gastroenteritis, frequently including bloody diarrhea, is observed (9). Cases of HUS caused by VTEC have been identified in all age groups but most frequently in infants and young children, and they are observed either during the course of outbreaks of VTEC infections or as sporadic cases. Contamination occurs via consumption of contaminated food, and most of the clinical signs observed are due to the absorption from the gastrointestinal tract of Shiga-like toxins (Stx) produced by the bacteria. Two types of Shiga-like toxins (also called verotoxins), Stx1 and Stx2, which presumably cause microangiopathic hemolytic anemia as a result of endothelial-cell injury, have been isolated. Other bacterial virulence factors may play a role in the pathological process, including an outer membrane protein, intimin, the product of the chromosomal gene *eae*, which is involved in bacterial adhesion to intestinal cells (6), as well as a plasmid-encoded enterohemolysin (Ehly) which has a cytolytic effect (20).

E. coli O157:H7 is the worldwide serotype of VTEC most commonly isolated from HUS patients. Other serogroups have been implicated (O26, O55, O103, O111, and O128) (3, 14, 17, 23), but their occurrence is likely to be underestimated, because isolation of non-O157:H7 VTEC still remains a challenge. Unlike most of the O157:H7 isolates, the majority of non-O157:H7 VTEC strains ferment sorbitol and therefore cannot be isolated by using media such as sorbitol MacConkey agar. Molecular biological and immunological techniques based on the detection of verotoxin genes and toxins, respectively, are so far the most reliable methods for detecting these pathogens in clinical specimens.

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Patients and clinical features. The average number of adults with HUS admitted to the medical intensive-care unit of the Clermont-Ferrand hospital used to be one every 18 months. (This hospital serves a large geographical area with approximately 1.3 million residents.) Between August 1996 and May 1997, this number increased considerably; 14 patients with clinical and biological evidence of HUS were admitted. In six cases, a VTEC strain was identified in the patients' stools by *stx*-specific PCR. The patients' mean age was 64 ± 19 years (range, 39 to 84 years). The male-to-female ratio was 1:5. All the patients developed HUS, defined as a Coombs-negative microangiopathic hemolytic anemia, thrombocytopenia without signs of disseminated intravascular coagulation, and acute renal failure (see Table 1). One of them (patient 1) had previously been admitted to the gastroenterology unit with severe abdominal pain and bloody diarrhea. Eleven days later, development of macroscopic hematuria and acute renal failure prompted her transfer to the intensive-care unit. Coombs-negative microangiopathic hemolytic anemia was defined as a hemoglobin level of <10 g/dl, intravascular hemolysis (serum haptoglobin, ≤0.1 g/liter), negative results of Coombs' test, and fragmented red cells and schistocytes on blood smear. Acute renal failure occurred in all the patients enrolled; four of them required renal replacement therapy. Fever (body temperature of >38°C) was present in four patients. Prodromal bloody diarrhea was observed in two patients, and nonbloody diarrhea was observed in four. All patients were treated with plasma exchanges, and none of them died. The mean number of plasma exchange treatments was 11 ± 2.

Isolation of VTEC strains by *stx*-specific PCR. Fecal samples were both cultured in Luria-Bertani (LB) broth (Difco Laboratories, Detroit, Mich.) and streaked out on Drigalski plates (Biomerieux, La Balme les Grottes, France), and they were then incubated at 37°C for 18 h. Bacteria from 1 ml of the LB broth culture or from at least 10 single colonies grown on Drigalski agar and previously suspended in 1 ml of saline were harvested, resuspended in 200 μl of sterile water, and incubated at 100°C for 10 min. Following centrifugation of the lysate, 10 μl of the supernatant was used in PCR. Oligonucleotides specific for amplification were 5′-ACCCTGTAACGAA GTTTGCG-3′ and 5′-ATCTCATGCGACTACTTGAC-3′ for

TABLE 1. General, biological, and clinical data of patients during the acute phase and characteristics of the *E. coli* strains isolated from patients' stool specimens

Characteristic	Patient[a]					
	1	2	3	4	5	6
Sex[b]	F	M	F	F	F	F
Age (yr)	45	84	63	39	76	77
Prodromic diarrhea[c]	+ (B)	+ (NB)	+ (NB)	+ (NB)	+ (B)	+ (NB)
Body temperature (°C)	38.5	37	38.5	38	39	37.2
Biological parameter						
Hemoglobin level (g/dl)	6.6	7.4	8.6	5.9	7.1	9.8
Haptoglobin level (g/liter)	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05
Schistocytes	+	+	+	+	+	+
Platelets (count/μl)	94,000	25,000	22,000	22,000	32,000	25,000
White blood cells (count/μl)	16,000	7,530	4,260	10,810	13,960	9,200
Creatinine (μmol/liter)	647	454	240	1,127	370	542
E. coli characteristic						
Serotype[d]	O6:H4	O91:H10	O91:H21	O rough:H16	OX3:H−	Ont:H−
stx[e]	stx_2	stx_2	stx_2	stx_2	stx_2	stx_2
ehly[e]	−	−	+	+	−	−
eae[f]	−	−	−	−	−	−

[a] +, present; −, not detected.
[b] F, female; M, male.
[c] B, bloody; NB, nonbloody.
[d] Ont, not O serotypeable.
[e] Stx- and Ehly-encoding genes detected by PCR and specific hybridizations.
[f] *eae* detected by dot blot hybridization.

stx_1 and 5'-ATCCTATTCCCGGGAGTTTACG-3' and 5'-GCGTCATCGTATACACAGGAGC-3' for stx_2 (4, 18). The PCR cycle included denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C, and extension for 1 min at 72°C (30 cycles) in a Perkin-Elmer Cetus DNA thermal cycler. Each of the primers was used at 0.125 mM, with 0.2 mM each deoxynucleoside triphosphate (Boehringer Mannheim, Meylan, France), 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM $MgCl_2$, and 1 U of *Taq* DNA polymerase (Appligène-Oncor, Illkirch, France). The reaction products were then analyzed by electrophoresis on 2% agarose gels after staining with ethidium bromide. DNA from the reference strain *E. coli* EDL 933 and a reagent blank, which contained all components except the template DNA, were included as positive and negative controls, respectively. The identities of the PCR products were then confirmed by Southern hybridization after transfer to Hybond N+ nylon membranes (Amersham International, Amersham, United Kingdom) and hybridization with a 1.1-kb *Bam*HI stx_1-specific or a 0.8-kb *Pst*I stx_2-specific DNA probe obtained from the recombinant plasmids pJPN37-19 and pNN111-19, respectively (16). DNA probes were labeled by random priming using the enhanced chemiluminescence system (ECL; Amersham International) according to the manufacturer's specifications, and hybridized filters were exposed to ECL-Amersham film. As shown in Fig. 1, PCR products of 584 bp were detected with the stx_2-specific primers with all stool specimens, but none of them gave a positive reaction with the stx_1-specific primers. Similar results were obtained by colony hybridation using Stx1- and Stx2-specific DNA probes (data not shown).

Bacterial identification and characterization. *stx*-positive isolates were identified biochemically by using an API 20E test (Biomerieux). All the isolates fermented sorbitol. Determination of their serotypes performed by the International *E. coli* and *Klebsiella* Reference Center in Copenhagen, Denmark, revealed that they belonged to different serotypes: O6:H4 (patient 1), O91:H10 (patient 2), O91:H21 (patient 3), O rough:H16 (patient 4), and OX3:H− (patient 5). The O-antigenic nature of the VTEC isolate from patient 6 could not be determined (O+:H−). Ehly-specific genes were detected by PCR using the primers 5'-CACACGGAGCTTATAATATTCTGT

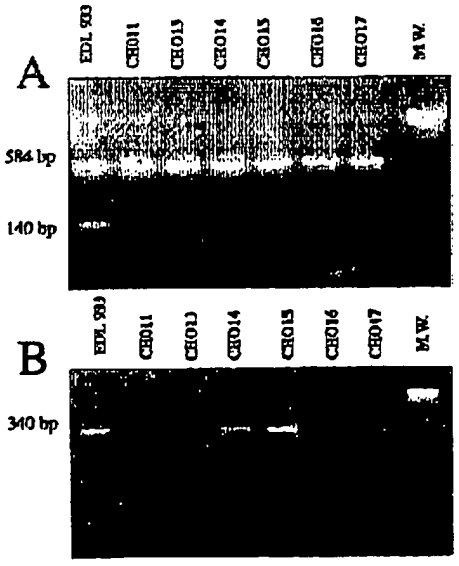

FIG. 1. Agarose gel electrophoresis of DNA fragments obtained by multiplex PCR with primers specific for stx_1 (140 bp) and stx_2 (584 bp) (A) and with primers specific for *ehly* (340 bp) (B) performed with genomic extracts from different *E. coli* strains: EDL 933, stx_1-, stx_2-, and *ehly*-positive O157:H7 reference strain; CH011, CH013, CH014, CH015, CH016, and CH017, isolates from patients 1 through 6, respectively. M.W., 1-kb ladder of molecular size markers (Boehringer Mannheim).

CA-3′ and 5′-AATGTTATCCCATTGACATCATTTGACT-3′. Conditions similar to those used for detection of *stx* genes were used, and the PCR products were identified by hybridization with a 3.4-kb *Hin*dIII fragment from pEO40 (20). Two strains, those isolated from patients 3 and 4, harbored Ehly-specific sequences as determined by PCR (Fig. 1) and hybridization; the same two isolates produced detectable hemolysis after 18 h of growth at 37°C on 5% washed sheep blood agar plates. The presence of *eae* was detected by dot blot hybridization; bacteria were grown in LB broth at 37°C overnight, and DNA was extracted by successive action of lysozyme, proteinase K, and Sarkosyl, followed by a purification step in a cesium chloride gradient. Hybridization was performed as described above by using a DNA probe specific for *eae*, i.e., a 1.4-kb fragment from an O157:H7 clinical isolate covering the entire *eae* open reading frame. DNAs from the reference strains *E. coli* EDL 933 and DH5α were included as positive and negative controls, respectively. None of the VTEC isolates hybridized with this DNA probe when they were tested under high-stringency conditions.

All the VTEC strains isolated in this study harbored Stx2-encoding genes. A higher prevalence of infection with VTEC producing only Stx2 among HUS patients has been reported in several investigations (10, 22). This may reflect the higher pathogenicity previously observed with Stx2- versus Stx1-producing strains both in in vitro assays with endothelial cells (13) and in murine models (24). All the bacterial strains were sorbitol fermenting, and none of them belonged to the O157:H7 serotype. However, although it is unlikely that we would have missed an O157:H7 isolate in the patients' stools, we cannot exclude the possibility of the occurrence of mixed infections with both a non-O157:H7 and an O157:H7 *E. coli* strain. Previous studies have described a few cases of mixed infections by detecting anti-O157 antibodies in patients' sera (2, 5). Unfortunately, we were not able to test patients' sera for anti-O157 antibody detection in this study. But if we had used routinely performed laboratory procedures with stool specimens, i.e., use of media such as sorbitol MacConkey agar or immunomagnetic separation techniques using anti-O157 antibody-coated beads, none of the present non-O157 isolates would have been detected. Analysis of their ribotype patterns (data not shown) did not reveal any homology, and they all belonged to different serotypes, indicating the sporadic nature of the cases. Three of them belonged to serogroups which have previously been associated with VTEC infections in humans (O91 and O6) (10, 12, 25) and isolated from meat and fecal samples of bovines in both the United States and Europe (15, 19). The O group OX3 is a provisional designation for a new O antigen, but a few isolates from this serogroup, differing from our isolate by the H antigen, have already been isolated from patients suffering from HUS in Europe. In Finland, an Stx2-positive *E. coli* OX3:H21 was detected in the stools of a 66-year-old woman, and in Denmark, *E. coli* OX3:H2 was detected in the urine of a patient (8, 11). Since strains belonging to this serogroup are detected in meat samples (19) and in domestic animals (1), they might represent another group of potentially life-threatening VTEC strains causing food infections.

Virulence factors other than toxins are likely to be required during the pathological process, including adherence factors and/or cytolysins. Among the six VTEC strains isolated in this study, none harbored the intimin-encoding gene (*eae*), which is involved in the attachment and effacing process, and Ehly sequences were detected in only two isolates. The presence of *eae* has mostly been described in O157:H7 isolates, but *eae*-negative non-O157 VTEC strains are also capable of causing disease indistinguishable from that caused by *eae*-positive O157:H7 (7, 11). It is likely that *eae*-negative VTEC strains pathogenic for humans may possess adherence factors other than Eae; investigations are currently being performed with isolates from this study in order to identify their adherence factors.

The role of the plasmid-encoded Ehly in the pathologic process of VTEC strains is not yet known. Ehly's produced by VTEC strains belong to the RTX (defined as repeats in toxin) toxin family and are closely related to the *E. coli* α hemolysin. They might act by lysing eucaryotic cells or by modulating the immune response, thus enhancing the virulence of VTEC. Previous studies demonstrated that patients infected with Ehly-positive VTEC were at a higher risk for developing HUS than patients infected with Ehly-negative strains (21). Only two bacterial isolates from this study harbored Ehly-encoding genes, indicating that synthesis of Ehly is not an absolute prerequisite for HUS development, although it might contribute.

From this study, we conclude that Shiga toxin-producing bacteria of serotypes other than O157:H7 can cause serious disease, as has been observed in several other instances. Cases of HUS due to non-O157:H7 *E. coli* are usually sporadic, unlike most of the infections due to serotype O157:H7. The reasons for this difference have not yet been addressed; it might be due to variations in the strains' virulence, but difficulties in identification of non-O157:H7 *E. coli* strains might also contribute to underestimation of their virulence potential. Although the cases of HUS observed in this study occurred in the same geographical area in a relatively short period (10 months), characterization of the VTEC isolates demonstrated that they were not related to each other. This might reflect an endemic situation, and since HUS represents the tip of an iceberg of clinical complications, it is likely that the number of mild infections is greatly underestimated. Development of diagnostic tools allowing detection of VTEC regardless of serotype is therefore urgently needed. Rapid and efficient detection of VTEC should be performed not only with patients suffering from HUS, but with anyone suffering from bloody diarrhea, in order to prevent both severe development of the disease and further spread of the pathogens.

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Epidemic of Gastrointestinal Tract Infection Including Hemorrhagic Colitis Attributable to Shiga Toxin 1-producing *Escherichia coli* O118:H2 at a Junior High School in Japan

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ABSTRACT. *Background.* An epidemic of gastrointestinal disturbances related to food ingestion occurred at a junior high school in Komatsu, Japan, and was caused by specifically Shiga toxin (Stx) 1-producing *Escherichia coli* O118:H2, which has not been reported previously in humans. No outbreak of *E coli*-producing Stx 1 alone had occurred.

Methods. A total of 526 students and 35 adult staff members who ate the same food at lunch in the school were investigated. Questionnaires about food consumption at lunch were given to all 561 subjects as well as to clinics and hospitals that had treated 79 patients. Stool specimens from 525 subjects, and food, water, and environmental specimens, including cooking utensils, were collected in an attempt to identify the pathogen.

Results. A total of 126 subjects (22.5%) developed a diarrheal illness. The pathogen was isolated from the stool in 131 subjects, 49 of which were asymptomatic, and from a dipper. Salads served over several days were identified as high-risk from food analysis. Gastrointestinal symptoms resembled those associated with previous infections of Stx-producing *E coli*, but were mild. No cases of the hemolytic–uremic syndrome developed. Headache was present in 87 patients. Three patients underwent surgery for acute appendicitis during this epidemic. Four of five carriers had received an antibiotic effective against the pathogen.

Conclusions. This outbreak of *E coli* O118:H2 demonstrated the clinical and epidemiologic features of infection by *E coli* that produces Stx 1 alone. Infections with such organisms are being recognized increasingly, and the pattern of disease observed may differ from the pattern observed with *E coli* O157:H7. *Pediatrics* 1999;103(1). URL: http://www.pediatrics.org/cgi/content/full/103/1/e2; *Escherichia coli O118:H2, Shiga-toxin 1, outbreak.*

ABBREVIATIONS. HUS, hemolytic–uremic syndrome; Stx, Shiga toxin; RPLA, reversed passive latex agglutination.

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In Japan from 1990 to 1995, seven outbreaks of gastrointestinal tract infection caused by *Escherichia coli* O157:H7 were reported.[1] In contrast, during the very short period from May to October 1996, 22 outbreaks of *E coli* O157:H7 occurred over a large area in Japan, and many people suffered from hemorrhagic colitis and hemolytic–uremic syndrome (HUS).[1] Although *E coli* O157:H7 is the most common source of Shiga toxin (Stx) 1 and 2, there are many other serotypes that also produce this toxin in vitro and in vivo. Four outbreaks of non-O157 Stx-producing *E coli* were confirmed in Japan from 1984 to 1995,[1] with the pathogen belonging to the serotypes O145:H-, O111:H-, and Out:H19. In other countries, only two previous outbreaks of infection by non-O157 Stx-producing *E coli*, such as O104:H21 in the United States [2] and O111:H- in Italy,[3] have been reported. All these isolates produced both Stx 1 and Stx 2, or Stx 2 alone.[1–3] Until the outbreak reported here, an outbreak of *E coli*-producing Stx 1 alone had not occurred. In July 1996, a large outbreak of exclusively Stx 1-producing *E coli* O118:H2 occurred in a junior high school in Komatsu city. This appears to be the first report in the world of clinical infection caused by this organism, and we describe here the clinical and epidemiologic features of the gastrointestinal infection caused by *E coli*-producing Stx 1, but not by Stx 2.

METHOD

Epidemiologic Investigation

An outbreak of a diarrheal illness occurred at R Junior High School between July 8 and July 21, 1996. This outbreak was first reported to the Minami Kaga Public Health Center, Ishikawa Prefectural Government, on July 15, 1996. Students and adult members of the school staff, all whom ate the same foods at lunch, were considered to be at risk and were investigated in detail. The food was prepared in the kitchen of the R Junior High School by professional cooks. Students and staff members ate lunch in an assigned classroom. A person was defined as a symptomatic subject if he or she developed at least one of the following symptoms: diarrhea (one or more watery or bloody stools in 24 hours or at least two loose stools in a 24-hour period), abdominal pain, vomiting, or headache.

A questionnaire was distributed to each student and adult staff member on July 15 and 19, 1996. It sought information about the foods eaten at lunch between July 4 and July 12, 1996; the symptoms; the date they appeared; and whether a physician was consulted. Information on the sequential changes of patients and the students who developed a gastrointestinal illness after July 20, 1996, was obtained from the teachers. A questionnaire also was

mailed to the 19 clinics and hospitals that were consulted by patients. It included questions about the date of onset of symptoms; date of consultation; specific symptoms; physical findings; results of laboratory tests used to evaluate the blood, urine and stool; and treatment administered. Of 79 such medical questionnaires, 75 were available for analysis.

Microbiologic Investigation

Between July 15 and 23, 1996, stool specimens were collected and examined for *Salmonella*, *Vibrio*, and *Staphylococcus*, using standard procedures. Representative stool specimens from 20 patients with diarrheal illness, including 5 patients with bloody diarrhea, also were examined for *Shigella*, *Campylobacter*, and *Yersinia*, using standard procedures. The first five colonies of *E coli* selected from MacConkey agar were serotyped using *E coli* O and H antisera, and sorbitol–MacConkey agar also was used in the routine screening for *E coli* O157:H7. These colonies of *E coli* also were examined for Stx 1 and Stx 2, using a reversed passive latex agglutination (RPLA) kit (Denka Seiken Co, Ltd, Tokyo, Japan).[4] Isolates of *E coli* were inoculated onto 10 mL of brain–heart infusion broth (Denka Seiken Co, Ltd) containing 900 μg of lincomycin. After overnight incubation at 37°C, colonies were inoculated to 1 mL of saline containing 5000 U of polymyxin B and shaken at 37°C for 30 minutes. The culture was centrifuged for 30 minutes at 3000 rpm. The supernatant was tested using an RPLA kit. This kit could detect 2 ng/mL of purified Stx. Representative specimens were reexamined for Stx by the polymerase-chain reaction procedure described previously.[5] Isolates were sent to the National Institute of Health (Tokyo, Japan) for serotyping. Asymptomatic subjects exhibited Stx-producing *E coli* in their stools were defined as healthy shedders. Immunoglobulin M antibody for O118 lipopolysaccharide in the serum of two patients with appendicitis was measured at the National Children's Medical Research Center (Tokyo, Japan).[6–9] Thirty samples of the foods served as lunch between July 8 and July 12, 1996, were stored at 4°C to investigate for pathogens in the event of an outbreak of gastrointestinal illness, and 9 samples of water and 29 environmental specimens obtained from utensils and other sources in the kitchen on July 15, 1996, also were examined by culture. Approximately 1 month after the onset of the present outbreak, stool culture studies were performed using the same method. Subjects exhibiting Stx-producing *E coli* in the reexamination of stools were defined as carriers. We inquired of carriers whether their family members developed gastrointestinal symptoms, and stools of family members were examined to isolate the Stx-producing *E coli*.

Statistical Analysis

Data are reported as means ± 1 standard deviation unit. The student's *t* test was used to compare laboratory values. The χ^2 test was used to test for differences in frequency distribution and proportion. The Yates' corrected χ^2 test was applied when the expected value for a cell was <5. A level of $P < .05$ was accepted as statistically significant.

RESULTS

The findings obtained from this outbreak are summarized in Table 1. Of 561 subjects who were at risk for the infection, 241 (43.0%) were defined as symptomatic and 126 (22.5%) developed a diarrheal illness. Of these patients, 9 were hospitalized with severe symptoms (6 patients with bloody diarrhea and 3 patients with acute appendicitis). The number of students with symptoms significantly exceeded that of the adult staff members (239/526 [45.4%] vs 2/35 [5.7%]; $P < .005$), and the number of students with diarrheal illness also significantly exceeded that of the adult staff members (125/526 [23.8%] vs 1/35 [2.9%]; $P < .01$). A characteristic of this outbreak was the surprisingly high number of patients who complained of headache, but there was no significant difference in the frequency of headache in the culture-positive versus culture-negative subjects (23/131 [17.6%] vs 59/394 [15.0%]). The majority of patients with abdominal discomfort complained of cramping in the periumbilical area. Only 5 (4.0%) of the 126 patients with diarrheal illness experienced vomiting. The average number of nonbloody diarrhea episodes per day was 3.3 ± 2.2 (ranging from 1 to 15 stools per day), and that of bloody diarrhea was 6.4 ± 2.6 (ranging from 3 to 10 stools per day). A watery diarrhea of 1.6 ± 0.9 days of duration (ranging from 0 to 3 days of duration) was antecedent to the onset of bloody diarrhea, and their peak body temperature was <38.0°C. No adult staff members developed bloody diarrhea.

TABLE 1. Summary of Findings: Epidemic of Gastrointestinal Illness at a Junior High School in Japan

	No.	(%)
People at risk	561	
Students (12½ to 15½ y)	526/561	93.8
Staff	35/561	6.2
Symptoms or signs	241/561	43.0
Asymptomatic	320/561	57.0
Symptomatic with nonbloody diarrhea*	117/561	20.9
Symptomatic with bloody diarrhea†	9/561	1.6
Symptomatic without diarrhea‡	115/561	20.5
Headache	87/241	36.1
With diarrhea	44/87	50.6
With abdominal pain only	34/87	39.1
Without other symptom	9/87	10.3
High temperature (>38.0°C)	5/241	2.1
Confirmed pathogen by RPLA	131/525‖	25.0
Asymptomatic	49/303	16.2
Symptomatic with nonbloody diarrhea	53/106	50.0¶
Symptomatic with bloody diarrhea	4/5	80.0¶
Symptomatic without diarrhea‡	25/111	21.6

* Nonbloody diarrhea with vomiting in 4 patients, headache in 40, and high temperature in 5.
† Bloody diarrhea with headache in 4 patients.
‡ Abdominal pain, vomiting, and/or headache without diarrhea.
‖ Stool culture was performed in 525 of 561 subjects at risk.
¶ $P < .005$ compared with the asymptomatic and symptomatic subjects without diarrhea.

Table 2 shows a comparison of laboratory findings during the acute phase in patients with bloody and nonbloody diarrhea. A mild but significant increase of the absolute neutrophil count and decrease of the platelet count were noted in patients with bloody diarrhea. Serum C-reactive protein value and leukocyte count were normal to slightly elevated in the majority of these patients. No fragmentation of erythrocytes was observed on the blood smears. Urinalysis revealed hematuria and/or proteinuria in 6 of the 29 patients tested. No cases of the HUS developed during the epidemic.

Two patients underwent surgery for acute appendicitis on July 16 and July 18, 1996. These patients exhibited previous watery diarrhea and a change from abdominal cramping to continuous pain in the right lower quadrant. Fever was absent in these patients. Their respective laboratory values were maximum leukocyte, 9.1 × 10^3 and 10.0 × $10^3/\mu L$ (9.1 × 10^9 and 10.0 × 10^9/L); and maximum C-reactive protein, <0.24 and 0.47 mg/dL (<2400 and 4700 μg/L). Macroscopic examination confirmed a hyperemic and swollen appendix in both patients. The ileocecal region also was involved in 1 patient, whereas serous ascites was seen in the other. Micro-

TABLE 2. Laboratory Findings During Acute Phase of Disease in Patients With and Without Bloody Diarrhea

	Bloody Diarrhea	Nonbloody Diarrhea	*P* Value
Leukocyte ($\times 10^3/\mu L$)	7.3 ± 1.4 (9)	6.4 ± 2.0 (15)	.254
Neutrophil ($\times 10^3/\mu L$)	5.5 ± 1.7 (8)	4.0 ± 1.1 (11)	.028
Hemoglobin (g/dL)	14.3 ± 1.1 (9)	13.6 ± 2.2 (15)	.388
Platelet ($\times 10^4/\mu L$)	23.2 ± 6.7 (9)	28.9 ± 5.9 (15)	.039
CRP (mg/dL)	0.6 ± 0.3 (9)	0.5 ± 1.1 (11)	.784
Blood urea nitrogen (mg/dL)	10.7 ± 4.7 (9)	12.0 ± 2.7 (10)	.450
Creatinine (mg/dL)	0.8 ± 0.2 (8)	0.6 ± 0.1 (10)	.035
LDH (U/L)	348.8 ± 59.3 (9)	354.4 ± 38.3 (10)	.807
AST (U/L)	19.8 ± 7.6 (8)	17.5 ± 3.9 (11)	.401
ALT (U/L)	16.9 ± 18.2 (8)	11.2 ± 2.6 (11)	.315

The number of patients is noted in the parentheses. CRP indicates C-reactive protein; LDH, lactate dehydrogenase; AST, asparate aminotransferase; ALT, alanine aminotransferase. For conversion to SI units: leukocyte count, $1/\mu L = 1 \times 10^9/L$; hemoglobin, 1 g/dL = 0.155 mmol/L; platelet count, $1/\mu L = 1 \times 10^9/L$; CRP, 1 mg/dL = 1×10^4 μg/L; blood urea nitrogen, 1 mg/dL = 0.375 mmol urea/L; creatinine, 1 mg/dL = 88.4 μmol/L; LDH, AST, and ALT, 1U/L = 1U/L.

scopic examination revealed hemorrhage and necrosis of an edematous appendicular mucous membrane. Slight infiltration of the appendix by neutrophils was present in only 1 of the 2 patients. Although no pathogen was isolated in cultures of the specimens of resected appendix, immunoglobulin M antibody for O118 lipopolysaccharide was present in the serum of both patients. In another 1 patient who underwent appendectomy on July 20, 1996, close examinations for pathologic changes of the appendix and causal relationship between Stx-producing *E coli* and appendicitis were not conducted.

As confirmed by the examination of 10 representative samples using the polymerase-chain reaction procedure, exclusively Stx 1-producing *E coli* was isolated by RPLA from stool specimens of 131 (25%) of 525 subjects whose stools were examined by culture, and the incidence of pathogen isolated from subjects with diarrheal illness was significantly higher than that for asymptomatic and symptomatic subjects without diarrhea (Table 1). Of 131 subjects found to be positive for Stx-producing *E coli*, 57 (43.5%) developed a diarrheal illness, 25 (19.1%) were symptomatic without diarrhea, and 49 (37.4%) were asymptomatic. There was no significant difference in the incidence of detection of this pathogen in the students versus the adult staff members (127/490 [25.9%] vs 4/35 [11.2%]). The isolate was identified as *E coli* serotype O118:H2. This isolate fermented sorbitol. Additional characterization of the strain, such as the ability to adhere to epithelial cells or possession of the eae gene, was not performed in this study.

Although Stx-producing *E coli* was not isolated from the samples of food and water, it was isolated from a dipper and identified as O118:H2. The analysis of the food eaten by the culture-positive versus culture-negative subjects revealed that high-risk food items served as lunch were coleslaw salad (July 5, 1996; $P < .005$), chicken and cucumber with cold mustard sauce (July 8, 1996; $P < .05$), sour sauce salad (July 9, 1996; $P < .025$), egg salad (July 10, 1996; $P < .005$), and corn salad (July 11, 1996; $P < .01$). Other food items served as lunch were rice; bread; soup; packed sterile milk; and thoroughly cooked meat, fish, eggs, and vegetables. No subjects developed the infection at the 11 other schools that had served the same vegetables and other foods and used the same menu. Five cooks developed no gastrointestinal symptoms, and Stx-producing *E coli* was not isolated from their stools.

Of 75 symptomatic patients who consulted a hospital or clinic, 56 received antimicrobial agents. New quinolones were used in 33 patients (norfloxacin, 17; enoxacin, 5; lomefloxacin, 4; levofloxacin, 4; tosufloxacin, 2; and ciprofloxacin, 1); fosfomycin in 27; macrolides in 3 (clarithromycin 2 and josamycin 1); cephems in 3 (cefaclor, cefteram piroxil, and cefuroxime axetil); and tetracyclines in 1 (minocycline). Eleven patients received two antibiotics in combination. Duration of antibiotic therapy was 4.5 ± 2.4 days. *E coli* O118:H2 was susceptible to these antibiotics. Table 3 shows the relationship between the isolation of pathogen from the stool and the duration of antibiotic treatment. Although effective agents were used in treating this pathogen, 9 of 15 patients still exhibited it 1 day after the administration of antibiotics. In fact, the pathogen was detected even after the administration of antibiotics for 2 or 3 days.

Table 4 shows the results of reexamination of stools from 470 students and 32 adult staffs for Stx-producing *E coli*. In the 49 asymptomatic subjects identified as healthy shedders by the first stool culture, the pathogen disappeared from their stools without treatment on days 28.6 ± 5.1 after the first culture. However, reexamination of the stools of symptomatic subjects on days 26.0 ± 5.4 after the

TABLE 3. Detection of Stx1-producing *E coli* in Stool Cultures After the Administration of Antibiotics

Days after Administration		No. of Cases	Antibiotics Used
1 (15 Cases)	Pathogen (+)	9	N4, F3, N + F2
	Pathogen (−)	6	F2, M2, N1, N + F1
2 (4 Cases)	Pathogen (+)	2	N1, C1
	Pathogen (−)	2	N2
3 (2 Cases)	Pathogen (+)	1	N1
	Pathogen (−)	1	N1
4 (2 Cases)	Pathogen (+)	0	
	Pathogen (−)	2	N1, M + N1

Pathogen (+) indicates confirmed pathogen in stool; pathogen (−), no confirmed pathogen in stool; C, cephems (cefteram piroxil); F, fosfomycin; M, macrolides (clarithromycin and josamycin); N, new quinolones (norfloxacin, levofloxacin, lomefloxacin, and enoxacin). Numbers to the right of these capital letters are the number of the patients using these antibiotics.

TABLE 4. Results of the Reexamination of Stools for Stx 1-producing *E coli* by RPLA

Result of First Stool Culture	No. of Cases† (502)	Confirmed Pathogen‡ (5)	(%) (1.0)
Asymptomatic subjects			
Pathogen (+)	49	0	
Pathogen (−)	236	0	
Total	285	0	0.0
Symptomatic subjects without antibiotic therapy			
Pathogen (+)	56	0	
Pathogen (−)	103	1	
ND	3	0	
Total	162	1	0.6
Symptomatic subjects with antibiotic therapy			
Pathogen (+)	25	2	
Pathogen (−)	17	2	
ND	13	0	
Total	55	4	7.3*

† The number of subjects whose stools were reexamined approximately 1 month after the onset of this outbreak.
‡ Confirmed pathogen by the reexamination of stools.
Pathogen (+) indicates confirmed pathogen by the first stool culture; pathogen (−), no confirmed pathogen by the first stool culture; ND, the first stool culture studies not performed.
* $P < .025$ compared with the symptomatic subjects without antibiotic therapy.

first examination showed that 5 subjects, 4 of whom had received effective antibiotics, exhibited Stx-producing *E coli*. These carriers were clinically healthy at the second stool culture. The pathogen disappeared from the stools of 5 carriers after the readministration of antibiotics. Stx-producing *E coli* was not isolated from stools of 22 family members of 5 carriers. Reexamination of stools showed that the incidence of pathogen from 55 symptomatic subjects treated with antibiotics significantly exceeded that of the 162 symptomatic subjects who did not receive antibiotics (Table 4).

In the present outbreak, although we could not identify patients with secondary infection, and we did not investigate whether the families of subjects at risk (except for 5 carriers) had gastrointestinal symptoms, 2 fathers of carriers were confirmed to develop a diarrheal illness on June 17 and 19, 1996.

DISCUSSION

There is no previous report of an epidemic or a sporadic occurrence of infection by *E coli* O118:H2. Furthermore, no outbreak of non-O157 *E coli* that produced a single toxin, Stx 1, had been reported previously in Japan[1] or in other countries.[2,3] We consider that infections caused by only Stx 1-producing *E coli* are becoming increasingly important because a small outbreak (the number of patients was 6) of *E coli* O26:H11-producing only Stx 1 occurred in Toyama prefecture, Japan, approximately 1 month after the present outbreak.[1] Attention has focused recently on Stx-producing *E coli* isolated from animals, because they are considered to be the primary source of this pathogen.[10-12] Fukui and associates isolated *E coli* O118:H16, which produced only Stx 1, from 2 of 7 calves with fatal infections in Shiga prefecture, Japan, between 1991 and 1993.[11] Garabal and co-workers also isolated *E coli* O118 from piglets with diarrhea.[12] Findings suggest that *E coli* O118:H2 may originate in domestic animals. On the other hand, *E coli* O118:H2, like other non-O157 Stx-producing *E coli*,[13] is not recognized by sorbitol–MacConkey agar used in the routine screening for *E coli* O157:H7, which does not ferment sorbitol.

In this outbreak, gastrointestinal symptoms were the same as those of the infections of Stx producing-*E coli* that were reported previously,[14-16] except that they were mild. Approximately 40% of the infected subjects became healthy shedders. Although mild abnormalities of urinalysis were observed, there were no signs and symptoms of HUS. This could be explained by the fact that the 50% lethal dose for mice of Stx 2 was 28-fold less than that of Stx 1,[17] and subjects had low susceptibility for Stx because they were not infants or elderly persons, but junior high school students and adult staff members. Although a case of HUS attributable to *E coli*-producing Stx 1 alone had been reported,[13] it can be expected that the prevalence of HUS attributable to exclusively Stx 1-producing *E coli* infection may be much lower than that of *E coli*-producing both Stx 1 and 2 or Stx 2 alone in infants or in the elderly. On the other hand, although the prevalence of diarrheal illness and other symptoms was significantly higher in the students than in the adult staff members, there was no significant difference in the incidence of isolation of *E coli* O118:H2 in those groups. This suggests that young people of junior high school age are more susceptible to Stx-producing *E coli* than are adults.

This outbreak was unusual in that many patients complained of headache. Because of Stx being referred to as a neurotoxin, headache is considered to be an effect of Stx. However there is a possibility that the headaches were not related to the infection of this organism, because there was no significant difference in the frequency of headache in the culture-positive versus culture-negative subjects. Another interesting feature of this outbreak was the finding of 2 patients with acute appendicitis. The diagnosis was verified surgically in both patients. However, additional discussion about the indication for operation is necessary, because the pathologic findings of the appendix in these patients resembled those seen with hemorrhagic colitis caused by infection of Stx-producing *E coli*.[14,18,19] Suppurative appendicitis was absent. Swelling of the appendix caused by Stx led to the symptoms of appendicitis. Appendicitis has likely occurred in other outbreaks and sporadic infections caused by Stx-producing *E coli*.

Although the apparent source of the primary infection was not identified, salad was considered to be a high-risk food in the analysis of the food eaten by the subjects. However, no subjects developed the gastrointestinal symptoms at the 11 other schools that had served the same vegetables as salad. The pathogen was isolated from a dipper used in this school. It was suspected that the infection may have been transmitted by placing uncooked, uncontaminated food in contaminated utensils. Undercooked, contaminated food was excluded as a source. Although the subjects affected in this outbreak were

not infants or the elderly, and *E coli* O118:H2 produced Stx 1 alone, the prevalence of the infection was distinctly high. This pathogen might have been consumed repeatedly in contaminated salads over several days.

The use of antibiotics for treating Stx-producing *E coli* infection is controversial. Carter and colleagues reported that antibiotic therapy was associated with an increased risk of secondary infection and a poor prognosis.[16] Karch and researchers demonstrated that incubating *E coli* O157:H7 with subinhibitory concentrations of trimethoprim-sulfamethoxazole resulted in a 4-fold increase in intracellular Stx and up to a 256-fold increase in extracellular Stx.[20] In the present outbreak, there was no evidence that the clinical course was exacerbated by the administration of antibiotics. However, it is questionable whether the antibiotics could eradicate the pathogen. Although this strain was susceptible to the antibiotics used in many clinics and hospitals, the pathogen was isolated from the stool of many patients even after the initiation of antibiotic therapy. Stool cultures performed ~1 month after the onset of outbreak indicated that the incidence of pathogen isolated from symptomatic subjects treated with antibiotics exceeded significantly that of symptomatic subjects not receiving antibiotic therapy. We consider that the number of carriers may be increased by antibiotic administration. However, Karch et al reported that 13% of patients with *E coli* O157 infection who received no antibiotic treatment became carriers.[21] These questions need to be answered to establish the appropriate treatment for Stx-producing *E coli* infections.

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Isolation and Characterization of Sorbitol-Fermenting Shiga Toxin (Verocytotoxin)-Producing *Escherichia coli* O157:H− Strains in the Czech Republic

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Two sorbitol-fermenting (SF) Shiga toxin-producing *Escherichia coli* (STEC) O157:H− strains were isolated from patients with hemolytic-uremic syndrome in the Czech Republic in 1995. Their phenotypic and genotypic characteristics and genomic DNA fingerprints were identical or closely related to those of SF STEC O157:H− strains isolated in Germany in 1988 to 1997. This indicates that the Czech isolates belong to the SF STEC O157 clone which is widespread in Germany. It is the first finding of the clone outside Germany.

Shiga toxin (verocytotoxin)-producing *Escherichia coli* (STEC) strains of serotype O157:H− (nonmotile) which ferment sorbitol and exhibit β-glucuronidase activity were first recognized in a 1988 outbreak of hemolytic-uremic syndrome (HUS) in Bavaria, Germany (9). Since then, they have been identified as a significant cause of HUS and diarrhea in Germany (6). Based on their phenotypic and genotypic features (1, 6, 12) and closely related pulsed-field gel electrophoresis patterns (10, 12), sorbitol-fermenting (SF) STEC O157:H− strains represent a new clone within *E. coli* serogroup O157 which shares pathogenic characteristics with non-sorbitol-fermenting (NSF) STEC O157:H7 (10, 12). Here we report the isolation of two SF STEC O157:H− strains in the Czech Republic. The objective of the study was to compare phenotypic and genotypic characteristics and to determine genetic relatedness of the Czech and German SF STEC O157:H− strains to find out whether the Czech isolates belong to the clone which is widespread in Germany.

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The Czech SF STEC O157:H− strains were isolated in August and October 1995 from two epidemiologically unrelated patients, aged 17 and 19 months, who were admitted to the University Hospital Motol, Prague, Czech Republic, for HUS preceded by bloody diarrhea. Although no NSF colonies were found in the patients' stool cultures on sorbitol MacConkey agar (SMAC), both patients had evidence of *E. coli* O157 infection. This was based on the presence of *E. coli* O157 antigen in their stools as detected by the *E. coli* O157 Antigen Detection enzyme-linked immunosorbent assay kit (LMD Laboratories, Carlsbad, Calif.) (15) and on significantly elevated titers of anti-O157 lipopolysaccharide antibodies in their sera (1:10,240 and 1:20,480) as detected by the indirect hemagglutination assay (3, 4). Slide agglutination of SF colonies with anti-O157 antiserum (ITEST, Hradec Králové, Czech Republic) and subsequent biochemical identification of such colonies revealed SF *E. coli* O157 strains in stool cultures of both patients. Serotyping by standard procedures (5) identified serotype O157:H−. The vehicle of infection was not determined for either patient.

Both *E. coli* O157:H− isolates were tested for fermentation of D-sorbitol and β-D-glucuronidase activity by tube tests (1), assayed for Shiga toxin 1 (Stx1), Stx2, and Stx2c production by the Vero cell neutralization tests (8, 13), and examined for enterohemorrhagic *E. coli* hemolysin (EHEC Hly) on enterohemolysin agar (2). Phage patterns were determined (14) and compared with those of German SF STEC O157 strains. The presence of stx_1, stx_2, stx_{2c}, *eaeA*, and EHEC *hly* genes was tested for by PCR procedures (10, 16, 17). Clonal relatedness of the isolates with German SF STEC O157 strains was determined by genomic DNA fingerprinting performed by randomly amplified polymorphic DNA PCR (RAPD PCR) with primer 1247 (7). The RAPD PCR profiles were visualized under UV light and photographed. A digital image of the gel was used to further analyze the profiles by the GelCompar software package (Applied Maths, Kortrijk, Belgium). Calculation of the similarity matrix was done by the Pearson product-moment correlation coefficient method (18). Hierarchic clustering was achieved by using the unweighted-pair-group method with the arithmetic averages clustering algorithm (18).

As shown in Table 1, the Czech SF STEC O157:H− isolates had identical phenotypic and genotypic characteristics which were at the same time identical with those of 24 German SF STEC O157:H− strains isolated in 1988 to 1996. The only exception was the absence of the stx_2 gene in isolate 230/95, which lost Stx2 production within 1 month after isolation, before it was genotyped. A new phage type designation (phage type 88) was assigned to the Czech and German SF STEC O157:H− isolates which shared a phage pattern that did not correspond to any of the previously recognized *E. coli* O157 phage types (14).

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TABLE 1. Phenotypic and genotypic characteristics of Czech SF STEC O157:H− isolates compared with those of German SF STEC O157:H− strains

SF STEC O157:H− isolate[a]	Sorbitol fermentation/ β-glucuronidase activity[b]	Result for[c]: Stx phenotype			Stx genotype			EHEC Hly production	EHEC *hly* gene	*eaeA* gene
		Stx1	Stx2	Stx2c	stx_1	stx_2	stx_{2c}			
Czech										
221/95	+/+	−	+	−	−	+	−	−	+	+
230/95	+/+	−	+[d]	−	−	−[d]	−	−	+	+
German[e]	+/+	−	+	−	−	+	−	−	+	+

[a] All isolates were phage type 88, a new phage type in *E. coli* serogroup O157.
[b] +, positive after 24 h.
[c] +, positive; −, negative.
[d] The isolate lost Stx2 production before genotyping.
[e] Characteristics of 24 isolates from 1988 to 1996 (based on references 1, 6, 9, 10, and 12, and on comparison of phage patterns of German and Czech strains).

RAPD PCR fingerprinting of genomic DNA (for a list and characteristics of the strains tested, see Table 2) showed that the Czech and German SF STEC O157:H− isolates had identical or closely related profiles that markedly differed from those of NSF STEC O157:H7/H− (Fig. 1). Analysis of the RAPD PCR profiles by the Pearson product-moment correlation method and by the unweighted-pair-group method with arithmetic averages clustering clearly distinguished three clusters of strains (Fig. 2). Nine SF STEC O157:H− strains from Germany and the Czech SF STEC O157:H− isolates gave a cluster with a nearly congruent pattern, thus showing high relatedness. The second cluster contained all NSF STEC O157:H7/H− strains; they were also closely related to each other but could be clearly distinguished from the SF STEC O157:H− strains. The third group consisted of two Stx-negative strains of serotypes O157:H19 and O157:H45; these strains were not related to either NSF or SF STEC O157:H7/H− (Fig. 2). Taken together with the other phenotypic and genotypic results, it can be concluded that the Czech SF STEC O157:H− strains belong to the clone which is resident in Germany.

This is the first report that SF STEC O157:H− strains belonging to the German clone can be a cause of HUS outside Germany. Although the vehicle of infection was not identified, the fact that none of the Czech patients had histories of travelling in Germany or consumption of foods imported from Germany makes domestic origin of infection very likely. Our findings thus suggest that the SF STEC O157 clone has begun to spread from Germany and that these strains can emerge as a public health problem in other countries. This has important diagnostic implications, emphasizing the need for diagnostic procedures which allow detection of infection with both NSF and SF *E. coli* O157 strains. In our study, combination of stool culture on SMAC with *E. coli* O157 stool enzyme-linked immunosorbent assay and anti-O157 serology enabled us to detect *E. coli* O157 infection despite the absence of NSF colonies on SMAC, thus aiming our diagnostic efforts towards searching

TABLE 2. List and characteristics of SF and NSF *E. coli* O157 strains analyzed by RAPD PCR fingerprinting

Strain	Serotype	Sorbitol fermentation[a]	Stx phenotype	Disease; origin[b] (reference[c])
1083-36/91	O157:H45	+	Stx negative	ID; G
693/91	O157:H19	+	Stx negative	ID; G
3010/96	O157:H7	−	Stx2	D; G
6651/96	O157:H7	−	Stx2	HUS; G
3075/96	O157:H7	−	Stx2c	D; G
EDL933	O157:H7	−	Stx1+Stx2	CDC (19)
3817/96	O157:H−	−	Stx1+Stx2	HUS; G
7579/95	O157:H−	+	Stx2	HUS; G
2260/96	O157:H−	+	Stx2	HUS; G
7713/95	O157:H−	+	Stx2	HUS; G
1995/96	O157:H−	+	Stx2	HUS; G
4162/94	O157:H−	+	Stx2	D; G
493/89	O157:H−	+	Stx2	HUS; G (10)
1996/96	O157:H−	+	Stx2	D; G
221/95/1[d]	O157:H−	+	Stx2	HUS; CR
703/88	O157:H−	+	Stx2	HUS; G (1)
1529/97	O157:H−	+	Stx2	HUS; G
221/95/2[d]	O157:H−	+	Stx2	HUS; CR
230/95	O157:H−	+	—[e]	HUS; CR
221/95[e]	O157:H−	+	Stx2	HUS; CR

[a] +, positive after 24 h; −, negative after 24 h.
[b] ID, infantile diarrhea; D, diarrhea; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.; CR, Czech Republic; G, Germany (nine German SF STEC O157:H− strains are representative isolates from 1988 to 1997).
[c] Strains for which no references are given are from this study.
[d] Sequential isolates from the same patient obtained on days 1 (221/95), 3 (221/95/1), and 4 (221/95/2) after admission.
[e] The isolate lost Stx2 production before RAPD PCR fingerprinting.

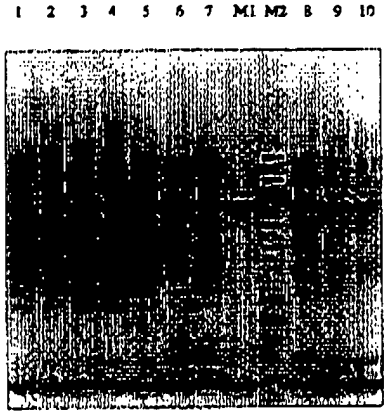

FIG. 1. Agarose gel showing RAPD PCR fingerprints of representative SF and NSF STEC O157 strains obtained with primer 1247. NSF *E. coli* O157:H7/H− strains 3075/96 (lane 1), 3010/96 (lane 2), EDL933 (lane 3), 6651/96 (lane 4), and 3817/96 (lane 5) are depicted. The SF *E. coli* O157:H− strains were Czech isolates 221/95 (lane 6) and 230/95 (lane 7) and German isolates 1529/97 (lane 8), 7713/95 (lane 9), and 1995/96 (lane 10). The molecular weight marker (M2) was DNA marker VI (Boehringer Gmbh); the molecular sizes of the fragments are (in base pairs) 2,176, 1,766, 1,230, 1,033, 653, 517, 453, 394, 298, 234, and 220. The internal standard (M1) consisted of a 1,600-bp and a 244-bp PCR product. In addition, internal standards were included in each lane.

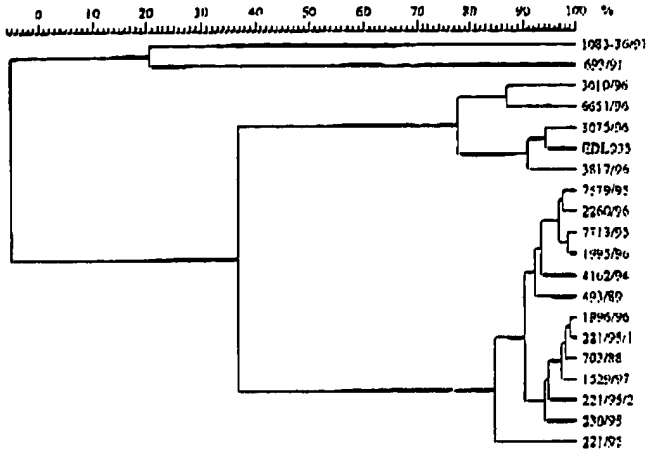

FIG. 2. Dendrogram derived from RAPD PCR data for Czech and German SF STEC O157:H− strains, NSF STEC O157:H7/H− strains, and SF Stx-negative *E. coli* O157:H19/H45 strains with the GelCompar software package. The characteristics of the strains are given in Table 2. The similarity scale is shown above the dendrogram (a similarity index of ≥80% indicates clonal relatedness).

for SF *E. coli* O157 strains. The procedures which have been successfully used to detect SF STEC O157 strains in German studies have included genetic methods (6, 9, 11) and the technique of immunomagnetic separation followed by plating magnetic particles with attached O157 bacteria on SMAC (11). Here, it should be remembered that SF STEC O157 strains, in contrast to NSF STEC O157:H7, do not grow on a selective cefixime-tellurite SMAC (11), since they do not tolerate high tellurite concentrations (12). Although the SF STEC O157:H− strains possess EHEC *hly* genes, no enterohemolytic phenotype could be observed (Table 1). This finding has consequences for the detection of such STEC in stool samples. While enterohemolysin agar plates have been successfully used for detecting EHEC Hly-producing NSF STEC O157:H7 (2), this method fails to detect nonhemolytic SF STEC O157:H− as characterized in this study. Consistent use of appropriate diagnostic methods for clinical and epidemiological studies is necessary to further evaluate significance of SF STEC O157 strains in human disease, to identify their reservoirs, and, based on that, to implement effective prevention of human disease.

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